A Dissertation

Entitled

Investigation of Skin and Skin Components Using Polarized Fluorescence and Polarized Reflectance Towards the Detection of Cutaneous Melanoma

By

Ye Yuan

Submitted as partial fulfillment of the requirements for

The Doctor of Philosophy in Engineering

Advisor: Dr. Patricia Relue

Graduate school

The University of Toledo

May 2006
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Ye Yuan

ENTITLED Investigation of Skin and Skin Components Using Polarized Fluorescence and Polarized Reflectance Towards the Detection of Cutaneous Melanoma

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ENGINEERING

Dissertation Advisor: Dr. Patricia Relue

Recommendation concurred by

Committee On Final Examination

Dean, College of Engineering
An Abstract of
Investigation of Skin and Skin Components Using Polarized Fluorescence and Polarized Reflectance Towards the Detection of Cutaneous Melanoma

Ye Yuan

Submitted as partial fulfillment of the requirements for
The Doctor of Philosophy in Engineering

The University of Toledo

May 2006

In an effort to investigate viability of using autofluorescence to detect superficial skin cancer (melanoma), polarized fluorescence spectroscopy was employed with the goal of reducing the contribution of the background fluorescence generated from the deep skin. Polarized reflectance was also employed to account for the effects of tissue scattering and absorption on the polarized fluorescence measurements and to measure changes in tissue scattering.

An investigation of the skin and its layers using polarized fluorescence spectroscopy has revealed that polarized fluorescence can be generated from the skin, the epidermis, and the dermis using polarized excitation light. The epidermis has the highest retention of fluorescence polarization, while the dermis has the lowest retention of fluorescence polarization. The dependence of the fluorescence anisotropy (a measurement of fluorescence polarization) on dermal thickness was measured, suggesting a role for
multiple scattering within the dermis in the depolarization of fluorescence in both the
dermis and the skin.

A hypothesis of NADH binding change resulting from a metabolic shift in cancer
cells was presented. An investigation of normal human melanocytes and melanoma cells
using fluorescence anisotropy of the NADH within the cells yielded results consistent
with this hypothesis. Normal melanocytes show appreciably higher fluorescence
anisotropy than melanoma cells.

Since dermal matrix erosion is one of the early stage events in cancer progression, an
experimental system was developed to mimic tumor invasion. The process of the
enzymatic erosion was investigated with polarized fluorescence, non-polarized
fluorescence and polarized reflectance spectroscopy to provide insight into the contrasts
between the normal and enzyme-digested dermal matrix. The degradation of the dermal
matrix with enzymes results in a decrease in fluorescence emission and light scattering in
the matrix. These results confirm the suggestion from literature studies with whole tissue
that collagenase-induced dissolution of the extracellular matrix is the cause of the
reduction of the fluorescence emission and scattering of the malignant tissue. Fluorescence
anisotropy, however, cannot detect the change in the dermal matrix induced
by enzyme digestion. It appears that enzymatic digestion does not change the
physicochemical properties of the remaining fluorophores and their microenvironment in
the dermis.
To my wife Wenli Fang, my kids,

my grandmother,

and my parents...
Acknowledgements

I would like to thank everyone who ever helped me in my life.

My Lord, Jesus Christ. He sent me to Toledo and guided me through my campus life here.

My family. They have always supported me, encouraged me, and shared with me.

Dr. Patricia Relue, my advisor. Dr. Relue introduced me into the wonderful world of fluorescence. I would not have achieved anything in my dissertation research without Dr. Relue’s guidance and help. I’m very grateful to Dr. Relue for offering me this opportunity to be trained in the exciting biophotonics field.

Dr. Brent Cameron who taught me optics and helped me through my dissertation research with his kind support with instrumentation and advice.

Tammy Phares who never minds bother and gave me kind help in the Bioprocessing Lab.

Drs. James Hampton, Jeffery Johnson, Jian-Yu Lu, Ronald Fournier, Vik Kapoor, Ezzatollah Salari, Scott Molitor, all of whom taught me various topics on class and off class. I’ve benefited from their teaching.

Bioengineering staff and students.

And off course, the people who helped fund this research. This research was supported by Whitaker Foundation and Skin Cancer Foundation.
# Table of Contents

Abstract iv  
Dedication vi  
Acknowledgments vii  
Table of Contents viii  
List of Figures xiii  
List of Tables xvii  
Chapter 1. Introduction and Background 1  
  1.1 Introduction 1  
  1.2 Melanoma Biology 2  
  1.3 Current Diagnosis of Melanoma 5  
  1.4 Light Propagation, Polarization, and Fluorescence 7  
    1.4.1 Polarization 8  
    1.4.2 Polarized Reflectance and Degree of Linear Polarization 9  
    1.4.3 Fluorescence 10  
    1.4.4 Polarized Fluorescence and Fluorescence Anisotropy 12  
  1.5 Current Research in Cancer Detection Using Autofluorescence 15  
    1.5.1 Melanoma Detection Using Autofluorescence 15  
    1.5.2 Problems Using Autofluorescence Methods in Skin 16  
    1.5.3 Cancer Detection Using Polarized Autofluorescence 18  
  1.6 Objectives of the Dissertation 19  
Reference 22  
Chapter 2. Polarized fluorescence spectroscopy of skin, epidermis, and dermis 26
List of Figures

Figure 1.1 — The typical progressional stages of melanoma development 4
Figure 1.2 — Jablonski diagram illustrating the electronic transitions associated with the generation of fluorescence 11
Figure 1.3 — Mechanisms of depolarization of fluorescence emission 13
Figure 2.1 — The structure of epidermis 27
Figure 2.2 — Schematic representation of the thickness of the skin layers (not drawn to scale) and light interaction within the skin 29
Figure 2.3 — Schematic diagram of the experimental set-up for fluorescence and reflectance spectroscopy 34
Figure 2.4 — Polarized fluorescence spectra of the epidermis, skin, and dermis generated using polarized excitation at 360nm 37
Figure 2.5 — Fluorescence anisotropy of the epidermis, skin, and dermis generated using polarized excitation at 350nm, 360nm, and 375nm 37
Figure 2.6 — Comparison of the average fluorescence anisotropies of the epidermis, dermis, and skin excited at 350nm, 360nm, and 375nm 38
Figure 3.1 — Photograph of a custom-designed fiber/filter holder 45
Figure 3.2 — Representative polarized fluorescence and polarized reflectance spectra from one sampling location of two dermis samples 48
Figure 3.3 — Fluorescence anisotropy and DLP of the dermis with increasing thickness 50
Figure 3.4 — Changes of the fluorescence anisotropy and DLP with the thickness of the dermis 54
Figure 4.1 — The pathway of glycolysis 61
Figure 4.2 — Tricarboxylic acid cycle (Krebs cycle) 63
Figure 4.3 — Schematic representation of the oxidative phosphorylation 64
Figure 4.4 — Malate-aspartate and glycerol-phosphate shuttles 65
Figure 4.5 — Schematic diagram of the digital imaging system 72
Figure 4.6 — Phase contrast and fluorescence images of human melanocytes, WM115 melanoma cells, and WM793 melanoma cells 77
Figure 4.7 — Representative fluorescence spectra of the melanocytes, WM115, and WM793 with polarized excitation at 360nm

Figure 4.8 — Fluorescence anisotropy of melanocyte, WM115, and WM793 cell suspensions

Figure 4.9 — Reflectance spectra of melanocyte, WM115, and WM793 cell suspensions at different cell concentrations

Figure 4.10 — Polarized reflectance spectra of the melanocyte, WM115, and WM793 cell suspensions at the concentration of $2 \times 10^7$cells/ml

Figure 4.11 — The average DLP of the backscattered light from the melanocyte, WM115, and WM793 cell suspensions with different cell concentrations

Figure 4.12 — Comparison of DLP between different cell types at the same cell concentrations and of DLP for the cell suspensions of $1 \times 10^7$cells/ml for the melanocytes and WM793 cells and $7 \times 10^6$cells/ml for WM115 cells

Figure 4.13 — Polarized fluorescence spectra of the melanocyte, WM115, and WM793 cell suspensions at the concentration of $2 \times 10^7$cells/ml

Figure 4.14 — Comparison of the fluorescence anisotropy of the cell suspensions with different cell concentrations and between different cell types

Figure 4.15 — Fluorescence ratio spectra of the different cell types

Figure 5.1 — The structure of dermis

Figure 5.2 — The hierarchical structure of collagen

Figure 5.3 — Schematic representation of the treatment of DED with enzymes

Figure 5.4 — Comparison of the average fluorescence spectra of the papillary and reticular sides of the DEDs excited at 350nm, 360nm, and 375nm

Figure 5.5 — Comparison of the normalized fluorescence spectra of the papillary and reticular sides of the DEDs excited at 350nm, 360nm, and 375nm

Figure 5.6 — Representative reflectance spectra collected from the papillary side of DED

Figure 5.7 — Comparison of the normalized ratio of fluorescence/reflectance spectra of the papillary (solid lines) and reticular (dashed lines) sides of the DEDs excited at (A) 350nm, (B) 360nm, and (C) 375nm

Figure 5.8 — Representative polarized fluorescence spectra of the papillary and reticular sides of the DEDs excited at 350nm, 360nm, and 375nm
Figure 5.9 — Fluorescence anisotropies calculated for each DED were averaged over the measurements of all 10 samples.

Figure 5.10 — Comparison of the average fluorescence anisotropies of the DEDs on the papillary side and the reticular side excited at 350nm, 360nm, and 375nm. (D) The average degree of linear polarization (DLP) of the backscattered light from both sides is shown.

Figure 5.11 — Fluorescence spectra of the DED samples for the control, 1 hr dispase treatment, 2 hr dispase treatment, and 3 hr dispase treatment.

Figure 5.12 — Fluorescence spectra ratios between dispase treated and control regions on each DED sample.

Figure 5.13 — Representative microscopic images (40×) of the PAS staining of the DEDs for 1 hr dispase treatment, 2 hr control area, and 2 hr dispase treatment area.

Figure 5.14 — Representative fluorescence spectra of the DEDs for control for dispase treatment; treatment with dispase for 1 hr and 2 hrs; control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for 2 hrs, 4 hrs, 8 hrs, and 16 hrs.

Figure 5.15 — Average fluorescence spectra ratios of the DEDs for control for dispase treatment; treatment with dispase for 1 hr and 2 hrs; control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for 2 hrs, 4 hrs, 8 hrs, and 16 hrs.

Figure 5.16 — Average fluorescence spectra ratios of the DEDs treated with dispase and collagenase replotted from Figure 5.15.

Figure 5.17 — The average ratio of total fluorescence intensity is shown as a function of total enzyme treatment time.

Figure 5.18 — Average fluorescence anisotropies of the DEDs as a function of excitation wavelength for control for dispase treatment; treatment with dispase for 1 hr and 2 hrs; control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for 2 hrs, 4 hrs, 8 hrs, and 16 hrs.

Figure 5.19 — Comparison of the average fluorescence anisotropy of the enzyme-treated areas with that of the control areas.

Figure 5.20 — Representative polarized reflectance spectra of the DEDs are shown for control for dispase treatment; treatment with dispase for 1 hr and 2 hrs; control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for 2 hrs, 4 hrs, 8 hrs, and 16 hrs.
Figure 5.21 — Comparison of the average DLP of the backscattered light from the enzyme-treated areas with that from the control areas for control for dispase treatment; treatment with dispase for 1hr and 2 hrs; control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for 2 hrs, 4 hrs, 8 hrs, and 16 hrs.  

Figure 5.22 — Comparison of average DLP with treatment time for the enzyme-treated areas and the control areas.
List of Tables

Table 1.1 — Visual characteristics of the ABCD rule for melanoma detection 5

Table 1.2 — Unassisted diagnostic accuracy of melanoma in the clinic based on ABCD rule 6

Table 2.1 — Excitation and emission maxima of the major fluorophores in the skin 30

Table 3.1 — Measured thickness of the dermis 46

Table 3.2 — Goodness of fit statistics of the curve fitting 54

Table 4.1 — Fluorescence properties of free and protein bound NADH 57

Table 4.2 — Summary of NADH binding with NAD-linked dehydrogenases involved in different types of metabolism 66

Table 4.3 — The melanocytes and melanoma cell lines under investigation in this study 73

Table 4.4 — Cell viabilities before the rotenone treatment and after the collection of fluorescence spectra 79

Table 4.5 — Change in fluorescence intensity for the different cell types after rotenone treatment 80

Table 4.6 — Effect of cell concentration on the peak wavelength (in nm) of the reflectance spectra of the settled cell suspensions 82

Table 4.7 — Cell viabilities before and after fluorescence and reflectance measurements of the three cell types 87

Table 4.8 — Fluorescence intensity ratio at 430 and 465 nm for the different cell types 91

Table 5.1 — The principle fluorophores of dermis 115
Chapter 1

Introduction and Background

1.1 Introduction

Cutaneous melanoma is the most deadly form of skin cancer and begins from melanocytes, which are normal pigment-producing cells in the epidermis of the skin. The incidence of cutaneous melanoma is increasing annually and the occurrence rate is one of the fastest of all cancers. According to the estimation of the American Cancer Society, there will be 62,190 new cases of cutaneous melanoma in the US in 2006, and about 7,910 patients will die of this disease [1].

Cutaneous melanoma is almost always curable at its earliest stages, but becomes notoriously resistant to any current medical treatment once it metastasizes to other organs. Thus, early detection of the disease is very crucial to saving patients’ lives. Physicians have practiced the ABCD rule to diagnose cutaneous melanoma [1], which is based on evaluation of “Asymmetry”, “Border”, “Color” and “Diameter” of a nevus. However, due to its subjectivity and high dependence on a physician’s experience, detection accuracy is low, and in many cases, benign nevi are removed by error [2]. Skin biopsy is so far the gold standard for diagnosis. This situation has prompted investigation and development of new and non-invasive methods for melanoma screening, with optical techniques emerging as promising tools.
Current investigations with optical microscopic imaging (dermoscopy) and multispectral imaging have applied ABCD rule-based pattern analysis to melanoma screening [3-9]. However, these methods have demonstrated low accuracy, a high number of false-positive cases, and inability to discriminate “featureless melanomas”. Autofluorescence (native fluorescence of tissue) has been demonstrated as a potential tool for melanoma detection [10-15], since it probes biochemical changes (i.e. changes in NAD(P)H fluorescence) associated with cancer development. However, the analyses of the ratios of the fluorescence intensities outside and inside nevi and fluorescence patterns in these investigations did not show consistent results [12, 13, 16], and the accuracy and reproducibility of autofluorescence methods need to be further improved. Since biochemical and morphological changes take place during the malignant transformation of a wide range of tissues [17], autofluorescence methods, which target naturally occurring fluorescent molecules associated with these changes, are potentially powerful tools for melanoma detection and deserve further study.

In the following sections, background information is given on melanoma biology and current methods of melanoma detection. In addition, mechanisms of light propagation and current research efforts using optical methods for cancer detection are presented. This chapter ends with several research questions and the goals of this dissertation.

1.2 Melanoma Biology

Cutaneous melanoma is a malignant skin tumor of melanocytic origin, characterized by its high invasiveness and metastasis. In normal skin, melanocytes dwell in the basal layer of the epidermis and generally do not proliferate. Each melanocyte establishes
contact with approximately 20-35 keratinocytes through its dendrites. Basal layer keratinocytes regulate melanocyte growth, morphology, proliferation, and expression of membrane-associated antigens through direct cell-cell contact [18]. A nevus is formed by the aggregation of benign melanocytic cells. Melanoma is thought to develop and progress in a sequence of five steps [19]: (1) congenital or common acquired nevi (benign nevi); (2) dysplastic nevi; (3) radial growth phase (RGP) primary melanoma; (4) vertical growth phase (VGP) primary melanoma; and (5) metastatic melanoma. However, melanoma may skip steps in its development.

The typical stages of melanoma progression are shown in Figure 1.1. The proposed progression from a melanocyte to a common acquired nevus appears to occur without genetic alterations. Benign nevus cells are structurally normal, with a finite life span and generally without cytogenetic abnormalities. Genetic changes are expected at the transition from a benign nevus to a dysplastic nevus, which is structurally atypical, persistent, proliferative, and is a suggested precursor for melanoma [19]. The progression from a dysplastic nevus to a RGP primary melanoma occurs gradually and spontaneously, probably without additional molecular changes [19]. Cells of RGP primary melanoma are incapable of metastasis but can individually invade the dermis. Additional genetic changes are expected at the transition from a RGP primary melanoma to a VGP primary melanoma, enabling the cells to invade the dermis as a large cluster of cells. Unlike RGP melanoma cells, VGP melanoma cells are competent for metastasis, are less dependent on exogenous growth factors, are capable of growth in vitro and on soft agar, and are tumorigenic in immunodeficient mice [19]. The progression from a
VGP melanoma to a metastatic melanoma may not require additional genetic changes [19].

Figure 1.1 The typical progressional stages of melanoma development shown from left, normal skin to right, metastatic melanoma (lack the stages of dysplastic nevus and RGP melanoma). Revised from [20].

Cell-cell interactions and cell-matrix interactions play important roles in melanoma progression. Numerous adhesion molecules in melanoma development and progression have been studied. Cadherins, which are cell surface glycoproteins mediating cell-cell adhesion, play an important role in skin homeostasis and melanoma development [21]. The cadherins have isotypes of E-, P-, and N-cadherin. In normal human skin, E-cadherin is expressed on the surfaces of keratinocytes, melanocytes and Langerhans cells. P-cadherin is expressed only on the surfaces of basal layer keratinocytes, and N-cadherin is expressed on the surfaces of stromal cells, such as dermal fibroblasts and vascular endothelial cells. E-cadherin is a major adhesion mediator between keratinocytes and normal melanocytes. The loss of E-cadherin expression on early stage melanoma cells
causes the loss of the control of keratinocytes over the melanoma cells, and the increased expression of N-cadherin on the melanoma cells allows the melanoma cells to adhere to each other and to the stromal cells, facilitating melanoma progression [21].

Another important cell-cell and cell-matrix adhesion receptor is the integrin \( \alpha_{v}\beta_3 \), which binds to a large number of extracellular matrix proteins [22]. The \( \beta_3 \) subunit of the \( \alpha_{v}\beta_3 \) integrin is up-regulated in melanoma progression, leading to increased invasiveness and tumorigenicity [21]. The \( \alpha_{v}\beta_3 \) integrin is thought to be the most specific melanoma-associated marker that can distinguish RGP from VGP melanomas [19].

1.3 Current Diagnosis of Melanoma

Due to their location in the superficial skin, melanocytic nevi can be evaluated directly with the naked eye. The so-called ABCD rule has been widely used by dermatologists to examine nevi for melanoma (Table 1.1).

| Table 1.1 Visual characteristics of the ABCD rule for melanoma detection*. |
|---|---|---|
| **Asymmetry** | Melanoma lesions | Benign nevi |
| Asymmetrical | round and symmetrical |
| **Border** | irregular | smooth, even |
| **Color** | multiple shades of brown, black | single shade of brown |
| Diameter | > 6 mm | < 6 mm |

* taken from [23].

However, the diagnostic accuracy of the ABCD rule-based examination of melanoma by dermatologists is low since it is subjective and highly dependent on the
dermatologist’s experience (Table 1.2). For each melanoma confirmatively diagnosed, about 20 to 30 benign lesions are referred to a physician and a large number of benign lesions (order of 10) are excised [24], causing unnecessary morbidity to patients. To improve the detection accuracy, dermoscopy (epiluminescence microscopy) has been used to enhance the diagnostic ability of dermatologists, enabling the evaluation of nevus features not observable to the naked eye [3, 5, 8, 25-27]. Dermoscopic examination of melanoma is still based on pattern analysis, such as border irregularity [28], pigment distribution [29], and number of colors present [24]. Dermoscopic examination of melanoma in some reports has achieved a slightly higher diagnostic accuracy (80% to 87%) [26, 28, 29] than unassisted examination, and has reduced the number of excised benign lesions [24, 25]. However, the performance of dermoscopic examination is still strongly influenced by the training and experience of dermatologists [25, 27], since dermoscopy depends on the appearance of features. Another disadvantage of dermoscopy is its inability to pick up early melanomas, such as melanoma in situ, RGP superficial spreading melanoma, small melanomas, and so-called featureless melanomas [5, 24, 30, 31]. Therefore, dermoscopy has thus far not significantly improved the diagnostic accuracy in the clinical management of the melanocytic lesions [32, 33].

<table>
<thead>
<tr>
<th>Physician’s experience</th>
<th>Diagnostic accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 10 years</td>
<td>80%</td>
</tr>
<tr>
<td>3-5 years</td>
<td>62%</td>
</tr>
<tr>
<td>1-2 years</td>
<td>56%</td>
</tr>
</tbody>
</table>

* data taken from [2].
In other efforts to improve melanoma detection, multispectral imaging, which takes advantage of acquiring both spectral and spatial information from a nevus, has been employed. Macroscopic spectral imaging has been applied to the collection of spectral images of atypical nevi with wavelength bands between 440nm and 700nm [34, 35]. The spectral signature of each pixel on an image is used to aid segmentation, and a spectrally segmented region is referred to excision for histologic examination [34, 35]. Multispectral imaging with wavelength bands within the visible to infrared region has also been investigated by another research group for the discrimination of melanoma from other pigmented lesions [6, 7, 9, 36]. Wavelength-dependent pattern analysis, such as lesion shape and color-related reflectance, has been applied with computer image analysis towards automatic melanoma detection. This multispectral imaging technique has achieved diagnostic accuracy comparable to that of an experienced clinician (80% sensitivity) [7, 9, 36]. However, since the ABCD rule-based analysis greatly depends on the lesion’s features, the diagnostic accuracy is not high, and a high number of false-positive cases result [7, 9]. Owing to the limits of pattern analysis-based optical imaging techniques, a need exists to explore a more objective, quantitative, and accurate way of detecting melanoma.

1.4 Light Propagation, Polarization, and Fluorescence

Human tissue is an optically turbid and absorbing media. A fraction of the light hitting the surface of a tissue reflects off due to a refractive index mismatch at the tissue/air interface. The remaining light penetrates into the tissue. Multiple scattering and absorption within the tissue attenuate the penetration of the light deeper into the tissue,
and multiple scattering is also a major cause of light backscattering from the tissue [37]. Unlike scattering, light absorption in tissue is an electronic transition process which results in heat, radiation (i.e. fluorescence), and photochemical reactions. Tissue absorption is dependent on the type of absorbers within the tissue and the wavelength of light. Light in the ultraviolet (UV) and infrared (IR) regions is readily absorbed in a tissue, while visible light with a wavelength below 600nm experiences both scattering and absorption in a tissue, resulting in the backscattering of 15 to 40% of the incident light [37]. The diffuse backscattered light bears information on tissue absorbers (i.e. bilirubin and hemoglobin) due to the absorption of the backscattered light by these absorbers, and thus, the diffuse backscattered light can be used for tissue diagnosis with optical techniques such as reflectance spectroscopy [37].

1.4.1 Polarization

The electric field vector of natural, unpolarized light vibrates in all planes perpendicular to the direction of propagation. In polarized light, the electric field vector is restricted to a single plane perpendicular to the direction of propagation. For partially polarized light, the polarization of the light, $P$, is defined as the fraction of the light that is linearly polarized, as shown in the following equation [38],

$$ p = \frac{P}{p + n} \tag{1.1} $$

where $p$ is the intensity of the polarized component, and $n$ is the intensity of the natural, unpolarized component. Tissue-light interaction such as multiple scattering results in
rapid depolarization of polarized light propagating in tissues. However, the backscattered light from certain tissues (including skin) retains a measurable degree of the initial polarization. In this situation, information on tissue and cell structures can be extracted from the degree of depolarization of the initially polarized light [37].

Birefringence is another phenomenon that affects the polarization state of light. Interaction of light with a birefringent material results in phase retardation of the light [39]. In tissues which are rich in fibrous structures such as collagen and muscle fibers, birefringence results from the linear anisotropy of these fibrous structures. The birefringence of a turbid tissue causes depolarization of an initially polarized light after multiple scattering events [39].

1.4.2 Polarized Reflectance and Degree of Linear Polarization

Light escaping from superficial tissue retains its original polarization state to some extent as it is only weakly scattered, whereas light from deep tissue completely loses its polarization state as it is strongly scattered. Polarization state, therefore, provides a means to differentiate the photons backscattered from the superficial tissue versus the deeper tissue sites. This loss in polarization with increasing scattering is important since early stage cancers develop in the superficial tissue. Conventional reflectance techniques collect all diffuse backscattered light from both superficial tissue and deep tissue sites. Polarized reflectance techniques can be used to reject the background light from deep tissue by collecting the diffuse backscattered light through a polarization gating, thus, minimizing the collection of depolarized light backscattered from the deep tissue and enhancing the information-bearing light from the superficial tissue. Imaging with
polarized reflectance has been shown to enhance the image contrast within the superficial skin [40, 41]. By calculating the degree of linear polarization from the polarized images collected, both the background signals from the deep skin and the common absorption of the superficial skin were shown to be reduced [40, 41].

Polarized reflectance spectroscopy has been used to study tissue scattering and absorption [42]. The degree of linear polarization, which is a measure of the retained initial polarization state, is calculated as,

\[ DLP = \frac{I_{r//} - I_{r\perp}}{I_{r//} + I_{r\perp}} \]

where \( I_{r//} \) is a parallel polarized reflectance spectrum and \( I_{r\perp} \) is a perpendicular polarized reflectance spectrum. The degree of linear polarization increases as scattering decreases [42]. However, strong absorption also yields a higher degree of linear polarization [42].

### 1.4.3 Fluorescence

Fluorescence is a luminescence phenomenon that occurs from the electronically excited states of fluorophores. The mechanism behind fluorescence is illustrated by the Jablonski diagram shown in Figure 1.2 [38].

The ground electronic state and the first and second excited electronic states of a fluorophore are denoted by \( S_0 \), \( S_1 \), and \( S_2 \), respectively. A fluorophore can exist in different vibrational energy levels within each electronic state, as denoted by 0, 1, 2, etc. When a fluorophore at its lowest vibrational energy level of \( S_0 \) is illuminated at an excitation wavelength (\( \lambda_{ex1}, \lambda_{ex2} \), etc.) within its absorption spectrum, absorption typically occurs. The absorbed energy excites an electron to some higher vibrational energy levels.
of either $S_1$ or $S_2$. The excited fluorophore rapidly relaxes back to the lowest vibrational energy level of $S_1$, a process called internal conversion. Fluorescence emission generally results from the lowest vibrational energy level of $S_1$ to a higher vibrational energy level of $S_0$. The fluorophore then quickly relaxes to the lowest vibrational energy level of $S_0$ through thermal equilibrium.

![Jablonski diagram](image_url)

**Figure 1.2** Jablonski diagram illustrating the electronic transitions associated with the generation of fluorescence. Light of wavelength $\lambda_{ex1}$ or $\lambda_{ex2}$ within the absorption spectrum of the fluorophore are absorbed and the energy decays to the lowest vibrational state of the $S_1$ state. From $S_1$, fluorescence may be emitted as the molecule returns to its lowest electronic energy state, $S_0$.

Due to the loss of energy in the relaxation to the lowest vibrational energy levels of $S_1$ and $S_0$, the energy of the emission light is lower than that of the excitation light, leading to emission of fluorescence at a wavelength longer than the excitation wavelength, a phenomenon called the Stokes’ shift. In addition to this characteristic, the fluorescence emission spectrum is typically independent of the excitation wavelength due to the rapid relaxation of fluorophores from higher electronic and vibrational levels to the lowest
vibrational level of $S_1$. Finally, the fluorescence emission spectrum is generally the mirror image of the absorption spectrum ($S_0 \rightarrow S_1$ absorption).

1.4.4 Polarized Fluorescence and Fluorescence Anisotropy

For a population of fluorophores with random orientation, excitation with unpolarized light results in the generation of randomly polarized fluorescence. However, when polarized excitation light is applied to the same population of fluorophores, polarized fluorescence emissions can normally be generated. This phenomenon is based on the existence of transition moments for the absorption and emission dipoles within a fluorophore, in which the emission is polarized along the emission dipole [38]. The fluorophores with absorption dipoles aligned parallel to the electric field vector of the excitation light have the highest probability of excitation. Those fluorophores with absorption dipoles aligned perpendicular to the electric field vector of the excitation light will not be excited. In general, the probability of absorption for a fluorophore is proportional to the square of the cosine of the angle between the absorption dipole and the direction of polarization of the excitation light as shown in Figure 1.3.

The degree to which the fluorescence emissions are polarized can be described quantitatively by the fluorescence anisotropy. The fluorescence anisotropy is defined as the ratio of the intensity of the polarized component of the fluorescence emission to the total intensity of the fluorescence emission, and is given by

$$ r = F_A = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad [38] $$
where $r$ (or $FA$) denotes fluorescence anisotropy, $I_{||}$ denotes fluorescence emissions oriented parallel to the polarization of the excitation light, and $I_{\perp}$ denotes fluorescence emissions oriented perpendicular to the polarization of the excitation light.

The fluorophores that are excited with polarized excitation light are distributed symmetrically around the direction of polarization of the excitation light, a phenomenon called photoselection [38]. Due to this photoselection, the maximum fluorescence anisotropy that can be obtained from a population of fluorophores with collinear absorption and emission dipoles is 0.4 [38]. For most fluorophores, an angular displacement of angle $\beta$ exists between the absorption and emission dipoles, resulting in a further decrease in fluorescence anisotropy (Figure 1.3).

![Figure 1.3 Mechanisms of depolarization of fluorescence emission. Photoselection results in the fluorescence emission being partially polarized to an extent that depends on the angular orientation of the absorption dipoles. An angular displacement between the absorption and emission dipoles leads to depolarization of the fluorescence emission. Brownian rotation of a fluorophore contributes to further depolarization of the fluorescence emission.](image-url)
In addition to the photoselection effect and the angular displacement of the absorption and emission dipoles, other factors contribute to the loss of fluorescence anisotropy. Rotational diffusion of a fluorophore is a common cause of the depolarization of fluorescence emissions (Figure 1.3), and this property makes fluorescence anisotropy a useful tool for studying the rotation ability of the fluorophore. The ability of a fluorophore to rotate reflects the physicochemical properties of the fluorophore and its microenvironment, such as the size and shape of the fluorophore, binding of the fluorophore to other molecules, and the viscosity of the solution. Resonance energy transfer (RET) of energy among fluorophores is also an effective mechanism of depolarization that occurs in concentrated fluorophore solutions. In turbid media, such as biological samples and tissues, light scattering and reabsorption of fluorescence emissions are also prominent causes of depolarization.

Since tissue scattering and absorption affect the measurements of both the fluorescence anisotropy and the degree of linear polarization, and since fluorescence photons experience the same scattering and absorption events as diffuse reflectance photons when backscattering from a tissue [43], polarized fluorescence and polarized reflectance techniques can be combined to extract useful information from a tissue. After accounting for tissue scattering and absorption with polarized reflectance, polarized fluorescence may provide useful information on the physicochemical properties of the fluorophores within the tissue. Combined use of polarized fluorescence and polarized reflectance spectroscopy has been reported to be capable of extracting intrinsic fluorescence (scattering and absorption-free) from the tissue [44].
1.5 Current Research in Cancer Detection Using Autofluorescence

Autofluorescence has been investigated extensively with various tissue types for its potential use in tissue diagnostics [17]. The autofluorescence signal comes from a special group of fluorescent molecules (fluorophores) which take part in cell metabolism (i.e., reduced nicotinamide adenine dinucleotide or NADH) or constitute the extracellular matrix of a tissue (i.e., collagen). The characteristics of these fluorophores may change with the malignant transformation of the tissue. Therefore, autofluorescence methods may provide a more objective, quantitative, and accurate way to detect cancer [45].

1.5.1 Melanoma Detection Using Autofluorescence

Fluorescence spectroscopy and imaging with autofluorescence have been investigated by several groups for their potential use for melanoma detection [10-16, 46]. In these studies, the cellular fluorophore NADH was targeted. Initial promising results reported by Lohmann et al. [10, 11] indicated that NADH fluorescence intensity was very small within the tumor region. They also observed that the fluorescence was considerably higher in the immediate surroundings of the tumor than it was in healthy skin. Benign nevi were reported not to follow the same pattern of fluorescence. This group of researchers defined the ratio of the maximum fluorescence intensity outside of the nevi to the minimum fluorescence intensity within the nevi as a metric for discrimination of melanoma from benign nevi [10, 11]. Unfortunately, the results were contradicted by two other research groups who tried to reproduce these experiments. These two groups concluded that no spectroscopically detectable intrinsic differences exist between the melanoma and normal tissue [12, 13, 16].
Using digital fluorescence imaging with excitation and emission wavelengths targeted at NADH, Chwirot et al. calculated the same ratio used by Lohmann et al. Using this ratio, they achieved about 83% sensitivity in melanoma detection, and concluded in situ melanoma detection using autofluorescence was a simple, objective, and useful tool for screening a large population of patients [14, 15]. Borisova et al. studied the fluorescence and reflectance spectra of compound nevi, dysplastic nevi, and melanoma, as well as normal skin [46]. The results of their studies showed that the fluorescence intensities within the spectral range measured were greatest for normal skin, followed by compound nevi, then dysplastic nevi, and finally melanoma. The melanoma showed very weak fluorescence due to significantly increased absorption within the malignant melanocytic lesion [46].

Due to the limited and sometimes contradictory results of the investigations with autofluorescence for melanoma detection, more work needs to be done to assess the accuracy and reproducibility of using fluorescence for lesion identification and classification. Meanwhile, the origin of the observed autofluorescence needs to be investigated to better understand mechanisms involved in the generation of fluorescence, and to better understand the variation expected when using fluorescence methods for melanoma detection [14, 15].

1.5.2 Problems Using Autofluorescence Methods in Skin

Multiple fluorophores exist in the skin. Major fluorophores in the epidermis are reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), keratin, and
several amino acids. Collagen and elastin are the major fluorophores in the dermis. Measured autofluorescence of the skin is a combination of the fluorescence of all excited fluorophores in the skin, and is further modified by the scattering and absorption of the skin.

Previous investigations have demonstrated that dermal fluorescence contributes to most of the skin autofluorescence generated, and that collagen fluorescence masks the fluorescence of NADH generated within the epidermis [43, 47, 48]. Since primary melanoma develops in the superficial skin, only fluorescence within the superficial skin (resulting from NADH and collagen in the basement membrane and papillary dermis) provides useful information for melanoma detection. However, background fluorescence generated in the deep dermis may mask the information-bearing fluorescence generated within the superficial skin when backscattered fluorescence is collected with conventional autofluorescence methods such as those reviewed above. Thus, this low signal to noise ratio makes it difficult to analyze the fluorescence changes of the fluorophores within the superficial skin, such as occur with NADH.

As described in Section 1.4.2, linear polarization can be applied to isolate the weakly scattered light escaping from superficial tissue from the multiply scattered light escaping from deeper tissue, thus enhancing the optical signal of the superficial tissue [41, 49-53]. The benefits of polarization may also be applied to autofluorescence methods (polarized fluorescence) to selectively enhance the detection of the fluorescence signals from the superficial tissue by reducing the background fluorescence from the deep tissue. The calculation of the degree of linear polarization or the fluorescence anisotropy may further cancel out the absorption effect of the tissue on the measured fluorescence. Furthermore,
polarized fluorescence can detect differences in the physicochemical properties of the targeted fluorophore and its microenvironment [38], providing an addition means for enhancing the contrast needed for melanoma detection using autofluorescence methods.

1.5.3 Cancer Detection Using Polarized Autofluorescence

Some pioneering work has been performed with polarized autofluorescence to detect tissue malignancy. Polarized fluorescence spectroscopy of cancerous and normal rat kidney tissues revealed a higher degree of polarization retained by the normal tissues than by the cancerous tissues [54]. The authors suggested that the flavins within the normal cells have less freedom of rotation possibly due to these fluorophores being more tightly bound to the enzymes and proteins within the normal cells [54]. However, with the same excitation wavelength of 488nm, the opposite trend was observed for human skin grade I squamous cell carcinoma. The squamous cell carcinoma showed a higher fluorescence anisotropy than the normal skin tissues, implying that either flavins are more tightly bound to proteins or keratin is in a more viscous microenvironment within the cancerous cells [55]. By targeting two native fluorophores using two excitation and emission wavelength pairs (340nm/440nm for NADH and 460nm/540nm for flavins), it was shown in both breast cancer and normal breast tissue that fluorescence anisotropy is dependent on tissue thickness [56]. For thin tissue slices (thickness < 30μm), the cancer tissue showed higher fluorescence anisotropy for both emission wavelengths. The authors suggested that energy transfer and/or rotational diffusion of fluorophores were more rapid in the normal tissue than in the cancer tissue. However, in thick tissue slices (i.e. 2mm), higher fluorescence anisotropy was seen in normal tissue (i.e. 2mm) than in cancer tissue;
the authors attributed this change to higher levels of multiple scattering in the cancer tissue that contribute to the depolarization of fluorescence [56]. In human rectal tissue polarized fluorescence spectra were also shown to minimize the characteristic absorption by blood and to reduce site-to-site variability in fluorescence intensity and spectral shape [57]. In the study, polarized fluorescence spectra, defined as the difference between parallel-polarized and cross-polarized fluorescence spectra, were compared. As the tissue absorption increased, the intensity of the polarized fluorescence spectra decreased [57]. By varying the angle made between the polarization axes of the analyzer and the excitation polarizer, polarized fluorescence was shown to be able to probe depth-resolved fluorescence from tissue phantoms and an oral tissue sample [58]. These angle-resolved polarized fluorescence measurements could decouple the epithelial and stromal fluorescence (excited at 340nm) of the oral tissue sample [58].

All of the investigations mentioned above measured polarized fluorescence from tissue samples, where various fluorophores such as extracellular matrix proteins might contribute to the measurements [56]. Separately probing the cellular component and the extracellular matrix component of a tissue with polarized fluorescence may provide insight into the source of fluorescence contrast between tissues.

1.6 Objectives of the Dissertation

Dermal collagen is a strong fluorophore and birefringent substance and contributes much background fluorescence from the deeper dermis to the measured fluorescence of the skin. Dermal collagen also rapidly randomizes polarized light. Background fluorescence emanating from the deeper dermis and the absorption effect resulting from
the pigmentation in the benign and malignant nevi make it difficult to differentiate the benign nevi from melanoma. Polarized fluorescence methods are expected to reduce the background fluorescence of the collagen from the deeper dermis and thus increase the contribution of the information-bearing fluorescence of the epidermis and the superficial dermis. The calculation of fluorescence anisotropy may remove the common absorption effect of superficial melanin, further facilitating the discrimination of pigmented skin lesions.

Several questions arise when techniques using polarized fluorescence are applied to the skin. First, can polarized fluorescence be generated from the skin with polarized excitation and how is the polarized fluorescence being generated from the different layers of the skin (epidermis and dermis)? Second, if melanoma cells in malignant nevi are metabolically different from nevus cells in benign nevi as is generally believed, can this difference be detected with polarized fluorescence? Third, can polarized fluorescence detect the changes in the dermal matrix induced by tumor invasion? Fourth, what are the mechanisms underlying the differences if any are found?

To answer the questions raised above, the research objectives of this dissertation are organized and described as follow:

In Chapter 2, an investigation of the polarized fluorescence from the skin and its two layers (epidermis and dermis) was carried out with polarized fluorescence spectroscopy. The results show that polarized fluorescence can be generated from the skin, the epidermis, and the dermis with polarized excitation. The epidermis has the highest retention of fluorescence polarization due to much less scattering within the epidermis.
Since prominent depolarization of fluorescence exists within the dermis, in Chapter 3, an investigation of the depolarization of fluorescence within the dermis was performed with polarized fluorescence spectroscopy to determine the dependence of the fluorescence anisotropy on the thickness of the dermis. As a reference, the depolarization of white light within the dermis was also investigated with polarized reflectance spectroscopy and is included in Chapter 3. Multiple scattering contributes to the depolarization of fluorescence and reflectance within the dermis as demonstrated by the dependence of the fluorescence anisotropy and degree of linear polarization on dermal thickness. The multiple scattering within the dermis contributes to the depolarization of fluorescence within the skin.

In Chapter 4, an assumption of NADH binding change resulting from the metabolic shift of cancer cells was presented and verified with an investigation of normal human melanocytes and melanoma cells using fluorescence anisotropy of the NADH within the cells. Normal melanocytes show appreciably higher fluorescence anisotropy than melanoma cells. This result is expected as the metabolic shift of the cancer cells to enhanced glycolysis leads to less NADH binding and a less viscous microenvironment for the NADH in the cancer cells.

In Chapter 5, the dermal matrix was eroded with enzymes to mimic tumor invasion. The process of the erosion was investigated with polarized fluorescence spectroscopy together with conventional non-polarized fluorescence and polarized reflectance spectroscopy to provide insights into the contrasts between the normal and enzyme-digested dermal matrix. The degradation of the dermal matrix with enzymes results in a decrease in fluorescence emission and light scattering in the enzyme-digested dermal
matrix. These results confirm the suggestion from former studies with whole tissue that collagenase-induced dissolution of the ECM is the cause of the reduction of the fluorescence emission and scattering of the malignant tissue [45, 59-62]. Fluorescence anisotropy, however, cannot detect the change in the dermal matrix induced by enzyme digestion. It appears that enzymatic digestion does not change the physicochemical properties of the remaining fluorophores and their microenvironment in the dermis.

Reference:


Chapter 2

Polarized fluorescence spectroscopy of skin, epidermis, and dermis

2.1 Introduction

2.1.1 Composition and Structure of Skin

Skin is comprised of epidermis, dermis, and hypodermis. As shown in Figure 2.1, the epidermis is generally divided into the stratum basale (the basal layer), the stratum spinosum (the prickle layer), the stratum granulosum (the granular layer), and the stratum corneum (the cornified layer) according to keratinocyte morphology.

The basal layer contains keratinocytes and melanocytes. Cuboidal keratinocytes in the basal layer provide new cells to the epidermis through mitotic activity and serve an anchoring function by attaching to the basement membrane. Melanocytes are normally anchored to the basement membrane with keratinocytes at a ratio of 1:5-8 [1]. Each melanocyte reaches into the upper layers of the epidermis with its dendrites and establishes contacts with approximately 20-35 keratinocytes, forming an “epidermal melanin unit” [2]. Melanocytes produce melanin, a pigment that provides photoprotection to the skin.

Overlying the basal layer is the prickle layer, in which keratinocytes are polyhedral in shape. Keratinocytes in this layer are not mitotic but are metabolically active.
The granular layer is comprised of flattened keratinocytes that are only briefly anabolic and gradually undergo catabolic destruction of intracellular organelles. The cornified layer, or the stratum corneum, consists of stacked dead keratinocytes with no nuclei and no metabolic activity. The cornified layer serves a barrier function for the skin.

Figure 2.1 The structure of epidermis. Distinct layers are shown from the top to the bottom: the stratum corneum, the stratum granulosum, the prickle cell layer, and the basal cell layer. Individual melanocytes are found along the basal layer. Taken from [3].

The dermis is separated from the epidermis by the basement membrane. The basement membrane functions as a semi-permeable barrier to prevent epidermal cells from invading the dermis and regulates material transfer between the epidermis and the dermis. The dermis mainly consists of collagen, elastin, and ground substance (glycosaminoglycans, water, and salt) and some cellular components. Collagen comprises about 77% of the fat free dry weight of skin [4]. Fibroblasts are the primary cell type in the dermis. Some organized structures also exist in the dermis, such as blood and
lymphatic vessels. The dermis can be further divided into two layers: the superficial papillary dermis and the deeper reticular dermis. The dermis is structurally and metabolically supportive to the epidermis. Underlying the reticular dermis is the hypodermis, which is primarily composed of fat. The hypodermis affords insulation, shock absorption and energy storage.

The thickness of the skin depends on the body location. As shown in Figure 2.2, the typical thickness of the epidermis is between 66μm and 178μm [5]. The thickness of the stratum corneum is between 8 μm and 15μm. The typical thickness of the dermis is from 1 to 4mm. The papillary dermis is comparable in thickness to the epidermis and is highly vascularized relative to the reticular dermis [6]. The subpapillary plexus at the junction of the two dermal layers and the deep dermal plexus at the junction of the hypodermis and the dermis provide vascular supply to the dermis [6].

2.1.2 Optical Properties of the Skin

The layered structure of the skin and the biochemical compositions therein impart distinct optical properties to each layer of the skin. Light propagation and distribution in the skin depends on the light absorbers (chromophores) and scatterers in the skin layers. Melanin, a major absorber in the epidermis, strongly absorbs short wavelength visible light and UV radiation. Melanin \textit{in vivo} has a maximum absorption at 335 nm, with a steep decline towards shorter wavelengths and a monotonic decrease towards longer wavelengths [7]. Other chromophores in the epidermis are primarily UVB-UVC-absorbing molecules, such as aromatic amino acids, DNA, and urocanic acid (UCA) [7]. Major absorbers in the dermis are hemoglobin, oxyhemoglobin, β-carotene, and bilirubin,
all of which are visible light absorbers [5]. Light scattering in the epidermis is due to particulate structures, such as nuclei, cell organelles and keratin fibers. In the dermis, collagen fibers cause multiple light scattering, and collagen fibers are also optically birefringent [5].

Figure 2.2 Schematic representation of the thickness of the skin layers (not drawn to scale) and light interaction within the skin. When a beam of incident light (1) strikes skin, part of it reflects off the skin surface (2), and the remaining part is refracted into the skin. Within the skin, light experiences either scattering (3) or absorption (4). When absorbed by a fluorescent molecule, fluorescence occurs (5).

Fluorophores in the skin are a special group of light absorbers that fluoresce upon absorption of excitation light. Major fluorophores in the epidermis are reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), keratin, and some amino acids. Collagen and elastin are the major fluorophores in the dermis. The excitation and emission wavelengths of the major fluorophores in the skin are shown in Table 2.1.

Measured skin fluorescence is a combination of the fluorescence of all excited fluorophores in the skin, and is further modified by skin scattering and absorption.
Previous investigations demonstrated that major skin fluorophores are located in the dermis, and collagen plays a major role in the formation of the fluorescence spectra of the skin [8-10].

<table>
<thead>
<tr>
<th>Fluorophores</th>
<th>Excitation maxima (nm)</th>
<th>Emission maxima (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavins (FAD)</td>
<td>450</td>
<td>515, 535</td>
<td>[11, 12]</td>
</tr>
<tr>
<td>NADH</td>
<td>290, 340, 351</td>
<td>440, 450, 460</td>
<td>[11, 12]</td>
</tr>
<tr>
<td>NADPH</td>
<td>336</td>
<td>464</td>
<td>[12]</td>
</tr>
<tr>
<td>Keratin</td>
<td></td>
<td>435</td>
<td>[13]</td>
</tr>
<tr>
<td>Collagen</td>
<td>325, 360</td>
<td>400, 405</td>
<td>[12]</td>
</tr>
<tr>
<td>Elastin</td>
<td>290, 325</td>
<td>340, 440</td>
<td>[12]</td>
</tr>
</tbody>
</table>

As illustrated in Figure 2.2, when light strikes the skin’s surface, a portion is reflected and the rest is refracted into the skin. The refracted light may be scattered or absorbed, or may excite fluorophores. The regular reflectance of a normally incident light beam on skin is between 4% and 7% over the entire spectrum from 250 to 3000nm, irrespective of skin pigmentation [5]. The penetration depth of light is wavelength dependant. Approximately half of incident light at the wavelength range of 320-400 nm (UVA) reaches the dermis [5]. The propagation and distribution of excitation light in the skin and the escape of fluorescence light from the skin are determined by the optical properties of the skin, such as the absorption coefficient $\mu_a$, the scattering coefficient $\mu_s$, and the anisotropy factor $g$. The absorption coefficient of the epidermis is greater than that of the dermis, but the thickness of the epidermis is significantly less than the thickness of the dermis. The scattering coefficients of the epidermis and the dermis are comparable. Both
the absorption coefficients and the scattering coefficients of the skin decrease with wavelength [14]. The anisotropy factor $g$ of the epidermis and the dermis are approximately equivalent and increase with wavelength [14].

2.1.3 Probing Superficial Skin Using Polarized Light

Linearly polarized light has been employed to extract the optical signal from the superficial skin where skin cancer begins [15-20]. Since the multiple scattering events within a biological tissue scramble the polarization of incident polarized light, polarization provides a means to differentiate the photons backscattered from the superficial versus the deeper tissue sites. Backscattered light from superficial tissue experiences less scattering events than backscattered light from deep tissue. Weakly scattered light retains its original polarization state to some extent, whereas multiply scattered light loses its original polarization state [16, 21-24]. Studies have demonstrated that for linearly polarized incident light, the backscattered light retaining its polarization scatters less than 10 to 15 times before escaping out from a highly scattering tissue [24-26].

In addition to multiple scattering, linear birefringence also contributes to the depolarization of incident polarized light [26]. Therefore, the incident polarized light is more rapidly depolarized in the dermis than in the epidermis due to the birefringent nature of the dermal collagen [17, 18]. Based on the difference in birefringence and scattering between the epidermis and the dermis, linearly polarized light has been shown to enhance image contrast in the epidermis and initial papillary dermis [16-18].
Polarized fluorescence will normally be generated with polarized excitation light. As described above, linearly polarized light can survive less than 10 to 15 scattering events in the skin before being totally randomized. Polarized fluorescence can thus be generated in the superficial tissue where the linearly polarized excitation light survives. The degree of polarization retained by the backscattered fluorescence is determined not only by the scattering and absorption of the tissue, but also by the rate of rotational diffusion of the excited fluorophores.

In this study, human skin and its two layers, epidermis and dermis, were studied using polarized fluorescence spectroscopy for the generation of polarized fluorescence from the native tissue fluorophores. To address the issue that multiple fluorophores exist in the skin, excitation with wavelengths of 350nm, 360nm, and 375nm were used in this study.

2.2 Materials and Methods

Epidermis and dermis samples were prepared from skin tissue. An experimental set-up for fluorescence and reflectance spectroscopy was employed to collect polarized fluorescence spectra from the epidermis, dermis, and skin samples.

2.2.1 Experimental Set-up

The experimental set-up for fluorescence and reflectance spectroscopy is shown in Figure 2.3. A 100W mercury arc lamp (Osram) was used as a light source. Excitation light at 350nm, 360nm, and 375nm was provided by a set of excitation bandpass filters (350nm±20nm, 360nm±20nm, and 375nm±20nm, Chroma Technology) within the
excitation filter wheel. A dichroic mirror (390nm) was used to reflect the excitation light to an optical fiber. Two silica-core optical fibers (Thorlab) with diameter of 1mm were used to deliver excitation light and collect fluorescence emissions. The distance of the illumination fiber tip to the sample surface was 1.5cm, leading to a circular illumination spot of 0.9cm in diameter. The distance of the collection fiber tip from the sample surface was also 1.5cm. The angle made between the two fibers was 45°. The first linear polarizer (polarizer) was a UV passing glass polarizer (Edmund Optics). The second linear polarizer (analyzer) was one of a pair of film polarizers (Edmund Optics) oriented orthogonally to one another. The film polarizers passed light of wavelengths greater than 380nm. A USB2000 spectrometer (Ocean Optics, FL) was used as a detector. Both the filter wheel and the spectrometer were controlled by a PC. Metamorph software (Universal Imaging Corporation) running on the PC was used to control the filter wheel. OOIBase32 operating software (Ocean Optics, FL) running on the PC was used to control the spectrometer. Fluorescence spectra collected by the spectrometer were displayed on the monitor and stored on the PC. To collect conventional non-polarized spectra, the linear polarizers were removed from the light path. For reflectance spectroscopy, a 6.5W LS-1 tungsten halogen light source (Ocean Optics, FL) with a neutral density filter (ND 1.0) was used. A silica-core optical fiber (Thorlab) with diameter of 1mm was used to deliver the halogen light. When collecting reflectance spectra, this fiber replaced the fluorescence illumination fiber and the light source was switched from the mercury arc lamp to the halogen light. Backscattered diffuse reflectance was collected using the same collection fiber for fluorescence emissions.
2.2.2 Preparation of Skin Samples

Skin tissue was obtained from a local tissue bank. Tissues were received from the tissue bank shortly after harvest and were received in growth media. Variation in skin, epidermis, and dermis thickness occurred as the skin tissue samples were taken from different sites on the body and from different donors. Prior to the collection of fluorescence spectra from the skin, the skin was cut into small pieces (1.5×1.5cm) and rinsed with PBS at least 3 times to remove traces of growth media. Skin samples used were cut from the central part of the skin sheet which was normally of uniform thickness to minimize variation in the thickness of the skin and skin tissue layers.

In addition to whole skin, fluorescence spectra were also collected from the isolated epidermis and dermis layers. The dermis was separated from the epidermis of the skin
using the method of Regnier [27-29]. Briefly, cadaver skin samples were cut into small pieces (approximately 2×2cm) and rubbed with a piece of cotton gauze soaked with 70% alcohol to minimize contamination. Each skin specimen was placed with the epidermis side up in a Petri dish (35×10mm) and covered with sterile calcium- and magnesium-free phosphate buffered saline (PBS, pH7.11) containing 0.25 μg/ml amphotericin B, 100 iu/ml penicillin, and 100 iu/ml streptomycin. The skin pieces were incubated at 37°C for 4 to 7 days. After incubation, the epidermis was gently peeled off the dermis with fine forceps, and both skin layers were analyzed spectroscopically.

2.2.3 Data Collection and Processing

The polarization orientation of the excitation light was set as parallel to the scattering plane, which is the plane containing the incident light beam and the backscattered light beam in the direction of detection. When collecting the fluorescence spectra, three individual spectra were collected and averaged within the spectrometer to enhance the signal to noise ratio (this setting with the spectrometer was used for all following studies). A total of 2 averaged spectra (technically 6 total spectra) were collected at the same location of each sample with each of the 3 excitation wavelengths and with both polarization configurations (parallel or perpendicular orientation). The spectra were taken sequentially with the polarization orientation between the polarizer and analyzer set as parallel, perpendicular, perpendicular, and parallel. The two parallel spectra were compared to determine if photobleaching occurred.

To calculate fluorescence anisotropy for dermis, epidermis, and skin, fluorescence spectra were imported to Matlab. Fluorescence anisotropy (FA) was calculated for each
sample at each excitation wavelength using Equation 1.3 and using the average of the two parallel and two perpendicular spectra collected. The FA was smoothed with a median filter if needed to suppress spike noise on the anisotropy spectrum caused by the spectroscopic measurement system. The average FA at each excitation wavelength was then calculated for the dermis, the epidermis, and the skin by averaging the FA over the samples of each tissue type.

### 2.3 Results and Discussion

Polarized fluorescence was generated and collected from all 3 sample types using linearly polarized excitation light at the 3 wavelengths. Representative polarized fluorescence spectra of the epidermis, the skin, and the dermis are shown in Figure 2.4. The fluorescence spectra shown are the average of the two fluorescence spectra collected with the same polarization orientation. Photobleaching was not observed during the collection of the spectra. The dermis yielded stronger fluorescence than both the epidermis and the whole skin. The skin yielded weaker fluorescence than the epidermis, most likely due to residual blood content in the skin samples acting as an absorber. The whole skin preparations were simply rinsed 3 times with PBS prior to spectra collection, whereas the skin samples used for isolation of the dermis and epidermis layers were incubated in PBS for 4 to 7 days. Thus, the residual blood content was gradually extracted from these skin samples.

The average fluorescence anisotropies were calculated for each excitation wavelength using the FAs calculated from 4 pieces of epidermis, 4 pieces of dermis, and 5 pieces of skin. The average fluorescence anisotropies for the three tissues are shown as a function
of excitation wavelength in Figure 2.5. The average FA for each tissue type show little dependence on the excitation wavelength used.

Figure 2.4 Polarized fluorescence spectra of the (A) epidermis, (B) skin, and (C) dermis generated using polarized excitation at 360nm. Spectra shown are the average of two spectra collected; integration time for each spectrum was 1500ms for the epidermis, 2000ms for the skin, and 600ms for the dermis.

Figure 2.5 Fluorescence anisotropy of the (A) epidermis, (B) skin, and (C) dermis generated with the same excitation wavelength are compared in Figure 2.6. Within the emission wavelength range of 420nm to 600nm, the highest fluorescence anisotropies were
obtained from the epidermis at all excitation wavelengths. In contrast, the dermis had the lowest fluorescence anisotropies at all excitation wavelengths. The fluorescence anisotropies of the skin were in between those of the dermis and epidermis. For 350nm excitation and with emission wavelengths greater than 580nm, the fluorescence anisotropy of the skin drops below that of the dermis; this drop is probably due to the error associated with a low signal to noise ratio.

![Figure 2.6 Comparison of the average fluorescence anisotropies of the epidermis (blue), dermis (magenta), and skin (green) excited at (A) 350nm, (B) 360nm, and (C) 375nm. The fluorescence anisotropies were averaged over several tissue samples as indicated by the panel legends. Data shown are the average +/- one standard deviation.](image)

In summary, polarized fluorescence of the skin and skin tissue layers could be generated and collected with our experimental setup. Polarized fluorescence intensity was highest from the dermis, followed by the epidermis and finally the whole skin. The magnitude of the fluorescence is dependent not only on the quantity of fluorophores present in the layer, but also on the scattering and absorption of the excitation and emission light. When comparing the fluorescence anisotropies of the tissues, the epidermis had the highest FA, followed by the skin and finally the dermis. The fluorescence anisotropy is a normalized measure that does not depend on the intensity of
the polarized emissions generated, but represents the degree to which the generated fluorescence has maintained the polarization state of the excitation light. Linearly polarized light is depolarized to a greater extent when the birefringence increases [26]. The dermis is more birefringent than the epidermis, thus, linearly polarized light is more rapidly depolarized in the dermis than in the epidermis [17, 18]. In addition, multiple scattering in the dermis is another major cause of the depolarization of both excitation and emission light. A recent study has shown that linearly polarized light has a physical penetration depth that is 6 to 7 times shorter in the connective tissue than in the epithelium [30]. Increased retention of the polarization state of excitation light in the epidermis relative to the dermis could lead to the generation of more polarized fluorescence in the epidermis than in the dermis. This is consistent with the results of our experiments. When comparing the FA of the skin to the epidermis, the lower fluorescence anisotropy of the skin could be caused by the contribution of both unpolarized excitation light and fluorescence backscattered from the dermis, with the excitation light yielding additional unpolarized fluorescence as it is backscattered out of the tissue.

Reference:


Chapter 3

Depolarization of fluorescence and reflectance within dermis

3.1 Introduction

As described in Chapter 1, depolarization of fluorescence emission is caused by photoselection of fluorophores, angular displacement of the absorption and emission dipoles, Brownian rotation of fluorophores, and resonance energy transfer among fluorophores. In biological samples, light scattering of both excitation and emission light is a major cause to depolarization [1].

Very little has been reported in the literature on the depolarization of autofluorescence by biological tissues. Depolarization of autofluorescence by both benign and malignant breast tissue was investigated by Mohanty et al. by measuring the dependence of fluorescence anisotropy (FA) on tissue thickness [2]. Excitation/emission wavelength pairs of 340nm/440nm and 460nm/540nm were used in this study. The fluorescence anisotropies measured at both emission wavelengths decreased with increasing tissue thickness, suggesting a contribution of multiple scattering to the depolarization of autofluorescence in these tissues [2]. For isotropic scattering, fluorescence anisotropy has been shown to decrease by 30% for a single scattering event for both excitation and emission light [3]. Light scattering in biological tissues is anisotropic with high values of the average cosine of scattering angle, $g$, and hence
depolarization is not as strong as for an isotropic media. The reduction of fluorescence anisotropy due to a single scattering event in breast tissue was shown to vary between 20-30% for \( g \) ranging between 0.7 to 0.9 [2].

In a preliminary study with our polarized fluorescence spectroscopy set-up, fluorescence anisotropy measurements of thick dermis (about 1mm) showed that the fluorescence anisotropies are dependent on the polarization orientation of the excitation light relative to the scattering plane. The scattering plane is defined as the plane containing the incident light beam and backscattered light beam in the direction of detection. The polarization orientation of the illumination light is said to be parallel to the scattering plane when the transmission axis of the polarizer is set to be parallel to both the scattering plane and tissue surface. The polarization orientation of the illumination light is said to be perpendicular to the scattering plane when the transmission axis of the polarizer is set to be perpendicular to the scattering plane (parallel to the tissue surface). When the polarization orientation of the excitation light was changed from parallel to perpendicular with respect to the scattering plane, the fluorescence anisotropy changed from positive to negative. When this same experiment was performed on a fluorescent agar gel (2% rhodamine in a 4% agar gel), the change in fluorescence anisotropy was not observed.

In chapter 2, it was suggested that multiple scattering within the dermis contributed to the depolarization of fluorescence of the skin. Fluorescence photons are generated through the electronic transition of fluorophores within the tissue. Diffuse reflectance is generated through the elastic scattering of the photons of incident light within the tissue.
Backscattered fluorescence photons experience the same optical scattering and absorption events in tissue as backscattered diffuse reflectance photons of the same wavelengths [4].

In this chapter, fluorescence anisotropy was measured as a function of dermal thickness to determine the effect of multiple scattering on fluorescence anisotropy in the dermis. As a reference, the degree of linear polarization (DLP) of the backscattered diffuse reflectance light was also investigated as a function of dermal thickness. With the polarization orientation of the illumination light either parallel or perpendicular to the scattering plane, spectra were collected such that the fluorescence anisotropy and the DLP could be calculated for the same locations in the tissue samples. By comparing the changes in these two quantities, it may be possible to determine if the changes in FA are due to the birefringence of the dermis.

3.2 Materials and Methods

Dermal samples with different thickness were prepared from skin tissue, and the thicknesses of the dermal samples were measured. The experimental set-up for fluorescence and reflectance spectroscopy used in Chapter 2 was modified and employed to measure the FA and DLP of the dermal samples to investigate the thickness dependence of these two quantities.

3.2.1 Experimental Set-up

The experimental set-up used for the collection of fluorescence and reflectance spectra is shown in Figure 2.3. A custom-designed fiber/filter holder (Figure 3.1) was
added to the experimental system that allowed reproducible positioning of the illumination and collection fibers, the polarizer, and the analyzer. With the fiber holder, the distance of both the illumination and collection fiber tips to the sample surface decreased from 1.5 to 1cm; the diameter of the circular illumination spot decreased to 0.5cm. The angle between the collection and illumination fibers remained 45°.

![Custom-designed fiber/filter holder](image)

Figure 3.1 Photograph of a custom-designed fiber/filter holder which was used to integrate the illumination (A) and collection (B) fibers, the polarizer (C), and the analyzer(D). Tissue samples on slides were placed on the support (E) that could be moved up and down to adjust the distance between the fiber tips and the tissue surface. A distance of 1cm was achieved by aligning the tissue surface with the rear bottom of the holder. The illumination light beam was centered in a hole in the support so that the light beam could pass through the hole to avoid any reflection from the support surface when measuring thin and relatively transparent tissue samples.

### 3.2.2 Preparation of Dermis

The dermis is very soft and elastic and is very difficult to cut into thin sections. However, when tissue samples are removed from a donor, the edges of the sample are thinner than the central portion. We took advantage of this and selected tissue from different locations within the sample to get dermis layers of different thicknesses. The dermis was separated from the epidermis of the skin using the method described in
Chapter 2. The dermis samples were cut into small pieces of 1-1.5cm × 1-1.5cm. To measure the thickness of the dermis, a piece of dermis was placed on a slide and covered with a coverslip. Since the coverslip is very thin and light, it does not squeeze the dermis by its own weight. The thickness of the “sandwich” was measured with a digital caliper (Mitutoyo, Japan) with a care to avoid squeezing the dermis. The thickness of the dermis was derived by subtracting the thickness of the slide and the coverslip from the total thickness of the “sandwich”. The measurement of thickness was performed at the left, middle, and right parts of the dermis, and the three measurements were averaged. A summary of the average thicknesses of dermis samples evaluated is shown in Table 3.1.

Table 3.1 Measured thickness of the dermis. Values given are average of three thickness measurements made with +/- one standard deviation.

<table>
<thead>
<tr>
<th>Dermis sample</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>2</td>
<td>153 ± 12</td>
</tr>
<tr>
<td>3</td>
<td>213 ± 25</td>
</tr>
<tr>
<td>4</td>
<td>243 ± 21</td>
</tr>
<tr>
<td>5</td>
<td>297 ± 55</td>
</tr>
<tr>
<td>6</td>
<td>363 ± 40</td>
</tr>
<tr>
<td>7</td>
<td>390 ± 56</td>
</tr>
<tr>
<td>8</td>
<td>453 ± 40</td>
</tr>
<tr>
<td>9</td>
<td>463 ± 35</td>
</tr>
<tr>
<td>10</td>
<td>577 ± 87</td>
</tr>
<tr>
<td>11</td>
<td>690 ± 35</td>
</tr>
<tr>
<td>12</td>
<td>843 ± 40</td>
</tr>
<tr>
<td>13</td>
<td>943 ± 116</td>
</tr>
<tr>
<td>14</td>
<td>1000 ± 60</td>
</tr>
<tr>
<td>15</td>
<td>1440 ± 46</td>
</tr>
<tr>
<td>16</td>
<td>1763 ± 25</td>
</tr>
</tbody>
</table>

3.2.3 Fluorescence and Reflectance Spectroscopy of Dermis

Polarized fluorescence spectra of the dermis were collected with 360nm excitation light. For the collection of polarized fluorescence and polarized reflectance spectra, the
polarization orientation of the illumination light was set as parallel or perpendicular to the scattering plane. Polarized fluorescence spectra were collected using the sequence described in Chapter 2. The polarized reflectance spectra were collected using a same sequence immediately after on the same location by switching the illumination fiber in the filter holder from the mercury arc lamp to the halogen light. Spectra were collected from a total of three sampling spots on each piece of dermis. Due to the size of the dermis pieces, the sampling spots partially overlapped. The fluorescence anisotropy and DLP were calculated for each sampling spot and were averaged over all 3 spots. Fluorescence anisotropy was calculated using Equation 1.3 and using the method described in Chapter 2. The DLP was calculated using Equation 1.2 and using the average of the two parallel and perpendicular polarized reflectance spectra.

### 3.3 Results and Discussion

Typical polarized fluorescence and polarized reflectance spectra of thin and thick dermis are shown in Figure 3.2. No photobleaching was observed with the fluorescence measurements. A prominent observation was that for the thick dermis (Figure 3.2C), the perpendicular polarized fluorescence spectrum was higher than the parallel polarized fluorescence spectrum with the polarization orientation of the excitation light perpendicular to the scattering plane. For all other cases shown, including the polarized reflectance of the thick dermis, the parallel polarizer/analyzer configuration had higher fluorescence and/or reflectance than the perpendicular configuration.
Figure 3.2 Representative spectra from one sampling location. (A, C) Polarized fluorescence and (B, D) polarized reflectance spectra of two dermis samples. (A, B) The thin dermis is 90µm; (C, D) the thick dermis is 1000µm. Legends give a pair of orientations for each curve, namely the orientation of the illumination polarization relative to the analyzer and the excitation polarization relative to the scattering plane. The integration times of the fluorescence spectra were 300 ms for the thin dermis and 30 ms for the thick dermis. The integration times of the reflectance spectra were 400ms and 200ms for the thin dermis with the orientation of the illumination polarization and scattering plane oriented parallel and perpendicular, respectively. The integration times of the reflectance spectra were 70ms for the thick dermis for both illumination orientations.

The fluorescence anisotropy and degree of linear polarization spectra were calculated for each sampling spot and then averaged over the 3 measurements for each dermis. The average FA and DLP of the 16 dermis samples of different thickness are shown in Figure 3.3. In each subplot, the FAs or DLPs are shown for illumination polarization oriented either parallel or perpendicular to the scattering plane.
For better comparing the changes of the fluorescence anisotropy and DLP with the thickness of the dermis, the mean fluorescence anisotropy with the emission at 440nm (fluorescence emission maximum of collagen cross-links) and the mean DLP with the wavelength at 440nm were plotted against the thickness of the dermis and are shown in Figure 3.4. The data curves were fitted with biexponential equations using the Curve Fitting Toolbox of Matlab, and the fit statistics was given in Table 3.2. The effects of multiple scattering within the dermis on the depolarization of both fluorescence and reflectance are clearly seen in Figure 3.3 and Figure 3.4. When the polarization orientation of the illumination light was set as perpendicular to the scattering plane, the fluorescence anisotropy changed to be negative with the thickness of the dermis above 150µm, whereas the DLP did not show similar change. The reason for this change is unknown. The depolarization of fluorescence due to multiple scattering was pretty fast with the thickness of the dermis increasing to about 300µm. As a reference, the depolarization of reflectance due to multiple scattering was very fast with the thickness of the dermis increasing to about 400µm. Within the dermis with thickness greater than 300µm and 400µm, respectively, multiple scattering no longer caused further prominent depolarization of the fluorescence and reflectance, leading to residual fluorescence anisotropy of 0.04 and a residual DLP of 0.15-0.18, with the polarization orientation of the illumination light set as parallel to the scattering plane. The residual fluorescence anisotropy and residual DLP were -0.02 and 0.12-0.15, respectively, with the polarization orientation of the illumination light set as perpendicular to the scattering plane.
Figure 3.3 Fluorescence anisotropy (left) and DLP (right) of the dermis with increasing thickness. The FA and DLP were calculated for polarization orientation of the illumination light either parallel or perpendicular to the scattering plane. The fluorescence anisotropy and DLP spectra were calculated for each of the overlapping sampling areas on each dermis piece and the average of the 3 spots is shown in the panels above, with the dotted lines representing ± one standard deviation.

Figure 3.4 Changes of the fluorescence anisotropy (left) and DLP (right) with the thickness of the dermis. These data summarize the panels given in Figure 3.3. Both the fluorescence anisotropy and DLP data were fit with biexponential curves.

Table 3.2 Goodness of fit statistics of the curve fitting.

<table>
<thead>
<tr>
<th></th>
<th>Fluorescence anisotropy - parallel</th>
<th>Fluorescence anisotropy – perpendicular</th>
<th>DLP - parallel</th>
<th>DLP – perpendicular</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSE*</td>
<td>0.00013</td>
<td>0.00042</td>
<td>0.0045</td>
<td>0.0080</td>
</tr>
<tr>
<td>ARS**</td>
<td>0.791</td>
<td>0.907</td>
<td>0.913</td>
<td>0.942</td>
</tr>
</tbody>
</table>

* Sum of squares due to error; ** Adjusted R-square.

Reference:

Chapter 4

Fluorescence Anisotropy of Cellular NADH of Cultured Melanocytes and Melanoma Cells

4.1 Introduction

Cellular NADH fluorescence has been widely used to study the metabolic activities of cells under various conditions. NADH is an important coenzyme in energy metabolism and its physicochemical properties are influenced by the metabolic changes in cells. Cancer cells have different metabolic properties than normal cells, which may change the binding and relative distribution of NADH in the cells. Fluorescence anisotropy, which is a fluorescence technique that targets the physicochemical properties of fluorescent molecules and their microenvironment, was studied with cellular NADH for its capability to detect the metabolic changes in cells.

4.1.1 Cellular NADH Fluorescence as a Tool to Study Cell Metabolic States, Proliferation, Differentiation, Transformation, and Drug Response

Under UVA (320-400nm) excitation, living cells yield fluorescence that is primarily generated by mitochondrial NADH and NADPH [1-8]. NADH and NADPH share very similar fluorescence properties, and therefore UVA-excited cell fluorescence is generally referred as NAD(P)H fluorescence. NADPH level is much lower than that of NADH in
the mitochondria [9, 10], and the fluorescence yield of NADPH is also substantially lower than that of NADH [11], therefore, the cell fluorescence is dominated by the mitochondrial NADH [9, 10, 12, 13].

Both free and protein-bound NAD(P)H are present in cells, and their properties have been studied by the methods of fluorescence spectral analysis [12, 14-16] and fluorescence lifetime measurements [3, 10, 14, 17-21]. Spectral fitting analysis by deconvoluting cell fluorescence spectra with reference fluorescence spectra of pure samples of endogenous fluorophores has been applied to study the relative contributions of free and protein-bound NAD(P)H to the cell fluorescence emission [22-24]. The fluorescence properties of the free and protein-bound NADH are summarized in Table 4.1.

<table>
<thead>
<tr>
<th>NADH type</th>
<th>Emission maxima (nm) *</th>
<th>Fluorescence life time (ns) **</th>
<th>Quantum yield ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>460</td>
<td>0.4-0.5</td>
<td>0.02</td>
</tr>
<tr>
<td>Protein-bound</td>
<td>440</td>
<td>2.0-2.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* taken from [25, 26]; ** taken from [20]; *** taken from [14]

Cellular NADH fluorescence was first studied by Chance and co-workers as an optimal indicator of intracellular oxidation-reduction states and an invaluable tool to study the ability of mitochondria to function in energy-linked processes [27, 28]. Since Chance’s pioneering work, cellular NADH fluorescence has been widely studied for its ability to probe cell metabolic conditions [19, 24], proliferation [29], differentiation [30], transformation [7, 8, 12, 16, 18, 22, 23], and drug response [31, 32].
Time-resolved fluorescence spectroscopy of yeast cells has revealed an appreciable decrease in the level of free NADH when applying oxygen to the cells, while the level of protein-bound NADH remained relatively stable [19]. The decrease of the free NADH in the yeast cells was attributed to the consumption of the free NADH through respiration during the transition from anoxia to normoxia. The change of the free NADH in the mitochondria could thus serve as an indicator of tissue oxidation-reduction state [19]. During the initial phase of cold hypoxia, hepatocytes showed an increase in fluorescence which resulted from the accumulation of NAD(P)H in the cells due to the perturbation of the electron transport chain by low oxygen concentration [24]. Spectral fitting analysis revealed a reduced ratio of free to protein-bound NAD(P)H in the cells during the initial phase of cold hypoxia, and the ratio of free to protein-bound NAD(P)H gradually increased during the prolonged period of hypoxia, suggesting an alternative anaerobic pathway was activated that reoxidized the NAD(P)H and produced NADH in the cytosol (free form) under this condition [24].

Fluorescence excitation spectra targeted at NADH emission have effectively distinguished rapidly growing cells from slowly growing cells in three cell systems [29]. The slowly growing cells showed lower intensity ratios at 320-350 and 370nm than their paired rapidly growing cells, suggesting a higher NAD level in the slowly growing cells [29].

Fluorescence spectral analysis of oral epithelial cells at distinct differentiation stages has distinguished terminally differentiated cells from less differentiated cells [30]. The terminally differentiated cells showed a blue-shift in the fluorescence maxima and a decrease in the fluorescence emission intensity. In addition, the terminally differentiated
cells also showed a loss of the excitation peak at 340nm on the fluorescence excitation spectra taken from the cells [30]. It appears that a low NADH level was associated with the terminally differentiated cells.

A decrease in NAD(P)H fluorescence with cell malignancy has been found in human keratinocytes [16], human bronchial epithelial cells [7], and human breast cells [8]. However, an increase in NAD(P)H fluorescence has been reported in transformed rat fibroblasts relative to normal rat fibroblasts [22]. The increased NAD(P)H fluorescence of the transformed fibroblasts resulted from a much higher NAD(P)H content in the transformed cells [22]. When excited at 365nm, leukemic cells also yielded stronger fluorescence emission than normal blood cells [23].

A blue-shift of NADH fluorescence spectra, suggesting an increase of protein-bound NADH, has been reported for malignant human keratinocytes [16]. However, a red-shift of NAD(P)H fluorescence spectra, suggesting an increase in free NADH, has been observed in human breast neoplasm cells [33] and transformed rat fibroblasts [22]. After correction for inner absorption, both rat liver cells and liver tissue slices demonstrated fluorescence emission maxima at around 440nm, similar to that of protein-bound NADH [12, 15]. However, Ehrlich tumor cells and hepatoma slices had fluorescence emission maxima close to 460nm, similar to that of free NADH in solution [12, 15]. Spectral fitting analysis has revealed a higher ratio of free to protein-bound NADH in transformed fibroblasts [22] and in leukemic cells [23] relative to their normal counterparts. The higher ratio of free to protein-bound NADH in the transformed fibroblasts was accompanied by an increased glucose consumption and a markedly higher lactic acid production that resulted from a higher lactate dehydrogenase activity [22]. However, the
normal fibroblasts demonstrated a markedly higher succinate dehydrogenase activity than the transformed ones, suggesting quite different levels of aerobic and anaerobic metabolism in these two cells [22].

Non-metastatic malignant cells have been shown to have higher average fluorescence lifetimes than metastatic malignant cells of various origins [18]. The higher fluorescence lifetimes might suggest a more hydrophobic environment of NADH in the non-metastatic cells [18]. NADH content was also consistently higher in the metastatic cells than in the non-metastatic cells [18].

NADH fluorescence has been used to study the drug response of cells [31, 32]. Cells treated with chemopreventive drugs showed a dose-dependent increase of redox ratio which resulted from a dose-dependent decrease of NADH fluorescence in the treated cells [31, 32].

4.1.2 Energy Metabolism in Cancer Cells May Change the Interaction of NADH with NAD-linked Dehydrogenases in the Cells

4.1.2.1 Brief Overview of Energy Metabolism

In living cells, energy metabolism consists of a number of components, including glycolysis, tricarboxylic acid cycle (TCA cycle or Krebs cycle), and oxidative phosphorylation. Glycolysis, an important part of both aerobic and anaerobic metabolism, occurs in the cytosolic compartment of a cell. As shown in Figure 4.1, glycolysis involves a series of reactions leading to the conversion of 1 molecule of glucose to 2
Figure 4.1 The pathway of glycolysis. The steps from glucose to pyruvate represent the glycolysis pathway and occur in the cytosol. The product pyruvate can be reduced by lactate dehydrogenase to lactate in the cytosol or oxidized by pyruvate dehydrogenase to acetyl-CoA in the mitochondria. Adapted from [34].
molecules of pyruvate. A total of 2 molecules of ATP and 2 molecules of NADH are produced from 1 molecule of glucose through glycolysis [34]. One binding site exists for NADH on glyceraldehyde-3-phosphate dehydrogenase in the glycolysis pathway. Although the enzymes of the glycolysis pathway are generally considered to be free in solution [34], several studies have demonstrated that some of the glycolytic enzymes are bound in dynamic equilibrium with the cytoskeleton [35-41]. A recent study, however, has shown no interaction of glyceraldehyde-3-phosphate-dehydrogenase with cytoskeletal elements [42].

In contrast to glycolysis, the TCA cycle and oxidative phosphorylation, two important pathways in aerobic metabolism, occur in the mitochondria of a cell. Pyruvate derived from glycolysis is transported into the mitochondria and converted to acetyl-CoA by pyruvate dehydrogenase, which is a multienzyme complex attached to the inner side of the inner mitochondrial membrane [34]. Acetyl-CoA is oxidized through the TCA cycle to yield NADH and FADH$_2$ as shown in Figure 4.2. The resulting NADH and FADH$_2$ are further oxidized through the oxidative phosphorylation pathway to produce ATP [34].

As shown in Figure 4.2, three binding sites exist for NADH on NAD-linked dehydrogenases, namely isocitrate dehydrogenase, $\alpha$-ketoglutarate dehydrogenase complex, and malate dehydrogenase, in the TCA cycle. The TCA cycle enzymes are located within the mitochondrial matrix, except for succinate dehydrogenase which is located on the inner surface of the inner mitochondrial membrane [34]. Studies on mitochondrial matrix organization have revealed that some of the TCA cycle enzymes, such as citrate synthase, malate dehydrogenase, fumarase, isocitrate dehydrogenase, and $\alpha$-ketoglutarate dehydrogenase complex can bind to the inner surface of the inner
mitochondrial membrane, and the TCA enzymes interact with one another [43]. Other studies have confirmed the binding of the complex I of the electron transport chain to mitochondrial NAD-linked dehydrogenases, including pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase complex, malate dehydrogenase, β-hydroxyacyl-CoA dehydrogenase [44], and isocitrate dehydrogenase [45]. The interactions between the complex I and the mitochondrial NAD-linked dehydrogenases imply their functions in facilitating metabolite transport and NADH channeling [45, 46].

![Tricarboxylic acid cycle](image)

**Figure 4.2 Tricarboxylic acid cycle (Krebs cycle). Adapted from [47].**

Oxidative phosphorylation is carried out by the electron transport chain and ATP synthase, both of which are located on the inner mitochondrial membrane as shown in Figure 4.3. The first step is the oxidation of NADH to reduce ubiquinone catalyzed by the complex I (NADH dehydrogenase) of the electron transport chain. FADH$_2$ is oxidized to
reduce ubiquinone by the complex II of the electron transport chain. The peripheral portion of the complex II is succinate dehydrogenase. Electrons are passed along the electron transport chain to molecular O$_2$ via a series of oxidations and reductions of the electron carriers, accompanying the transfer of H$^+$ from the inner mitochondrial matrix to the intermembrane space. ATP production is coupled with the influx of H$^+$ from the intermembrane space through the ATP synthase. Oxidation of the 2 acetyl-CoA molecules, which are derived from the 2 molecules of pyruvate, through the TCA cycle and oxidative phosphorylation yields 24 molecules of ATP [34]. One binding site exists for NADH on the complex I in the oxidative phosphorylation pathway (see Figure 4.3). Therefore, five binding sites exist for NADH within the mitochondria which are related to aerobic metabolism, and two of them, namely pyruvate dehydrogenase and the complex I, are attached to the inner mitochondrial membrane.

![Figure 4.3 Schematic representation of the oxidative phosphorylation. Complex I is NADH-ubiquinone oxidoreductase; Complex II is succinate-ubiquinone oxidoreductase; Complex III is ubiquinone-cytochrome-c oxidoreductase; Complex IV is cytochrome-c oxidase; Complex V is ATP synthase. Q and C represent mobile carriers, ubiquinone and cytochrome c. Adapted from [47].](image)
Under the condition of aerobic metabolism, cytosolic NADH generated from glycolysis is oxidized through mitochondrial oxidative phosphorylation to regenerate NAD for the continuation of glycolysis [34]. Due to impermeability of the inner mitochondrial membrane to NADH or NAD, two substrate shuttle systems, the malate-aspartate shuttle and the glycerol-phosphate shuttle, serve to transfer the cytosolic NADH.
reductive potential into the mitochondria [34]. Three molecules of ATP are produced from 1 molecule of cytosolic NADH through the malate-aspartate shuttle, and 2 molecules of ATP are produced from 1 molecule of cytosolic NADH through the glycerol-phosphate shuttle [34]. As shown in Figure 4.4, these two substrate shuttle systems provide two binding sites for NADH in the cytosol, one on the cytosolic malate dehydrogenase of the malate-aspartate shuttle and the other on the cytosolic glycerol-3-phosphate dehydrogenase of the glycerol-phosphate shuttle [34].

Table 4.2 Summary of NADH binding with NAD-linked dehydrogenases involved in different types of metabolism. Each enzyme has one site for NADH binding.

<table>
<thead>
<tr>
<th>Type of metabolism</th>
<th>Enzyme</th>
<th>Enzyme location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic metabolism</td>
<td>Glyceraldehydes-3-phosphate dehydrogenase</td>
<td>Free in the cytosol</td>
<td>[34, 42]</td>
</tr>
<tr>
<td></td>
<td>Lactate dehydrogenase</td>
<td>In the cytosol</td>
<td>[34]</td>
</tr>
<tr>
<td>Aerobic metabolism</td>
<td>Glyceraldehydes-3-phosphate dehydrogenase</td>
<td>Free in the cytosol</td>
<td>[34, 42]</td>
</tr>
<tr>
<td></td>
<td>Cytosolic malate dehydrogenase</td>
<td>In the cytosol</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>Cytosolic glycerol-3-phosphate dehydrogenase</td>
<td>In the cytosol</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>Pyruvate dehydrogenase complex</td>
<td>Attached to the inner side of the inner mitochondrial membrane</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>Isocitrate dehydrogenase</td>
<td>In the mitochondrial matrix; can bind to the inner mitochondrial membrane</td>
<td>[34, 43]</td>
</tr>
<tr>
<td></td>
<td>α-ketoglutarate dehydrogenase complex</td>
<td>In the mitochondrial matrix; can bind to the inner mitochondrial membrane</td>
<td>[34, 43]</td>
</tr>
<tr>
<td></td>
<td>Malate dehydrogenase</td>
<td>In the mitochondrial matrix; can bind to the inner mitochondrial membrane</td>
<td>[34, 43]</td>
</tr>
<tr>
<td></td>
<td>The complex I of the electron transport chain</td>
<td>Attached to the inner mitochondrial membrane</td>
<td>[34]</td>
</tr>
</tbody>
</table>

Under conditions of anaerobic metabolism, cytosolic NADH is oxidized to NAD by lactate dehydrogenase in order for glycolysis to proceed (Figure 4.1). This process yields
lactate and is called fermentation. Therefore, in anaerobic metabolism, two binding sites exist for NADH on NAD-linked dehydrogenases. In comparison, in aerobic metabolism, eight binding sites for NADH on NAD-linked dehydrogenases exist. The binding proteins associated with both aerobic and anaerobic metabolism are summarized in Table 4.2.

### 4.1.2.2 Energy Metabolism in Cancer Cells Leads to Less Binding of NADH with NAD-linked Dehydrogenases in the Cells

Increased rates of glycolysis and lactate production are characteristics of many if not all cancer cells [48]. Rapidly growing cancer cells can derive about 60% of their energy from glycolysis and 40% of their energy from oxidative phosphorylation [49], whereas normal cells derive less than 10% from glycolysis and more than 90% of their energy from oxidative phosphorylation [50]. Glycolysis and respiration are tightly coupled in normal cells. However, cancer cells lack the ability to integrate energy metabolism between glycolysis and respiration. As first revealed by Otto Warburg, aerobic glycolysis is a characteristic of many tumors [48]. Metabolic changes of cancer cells include enhanced glycolysis, excessive lactate production, and reduced respiratory capacity [22, 48, 51-58]. Underlying these metabolic changes are increased activities of glycolytic enzymes [22, 58-71] and decreased activities of TCA cycle and oxidative phosphorylation enzymes [22, 56, 60, 61, 66, 68, 72-80]. Mitochondria changes in rapidly growing cancer cells, such as fewer number, smaller size, fewer cristae and more dilute matrix [50, 66, 73, 75, 81], appear to be consistent with the shift of the cancer cells’ energy metabolism towards a high level of glycolysis. Fully transformed cells are most dependent on glycolysis and lactate production and least dependent on the
mitochondria for ATP synthesis [82], revealing an association between the progressively increased glycolytic rate and reduced respiration capacity with cancer progression [53, 67, 69, 82-84]. Aerobic glycolysis observed in most primary and metastatic cancer cells is constitutively up-regulated and is suggested to be the result of adaptation to consistent environmental pressures (i.e. hypoxia) in pre-malignant lesions [57].

Melanoma may favor aerobic glycolysis for energy production just as many other tumors do. Melanoma cells have shown elevated expression of c-myc oncogene in comparison to human melanocyte cell lines [85]. Activation of the c-myc oncogene results in an oncogenic transcription factor (c-Myc) which is capable of activating gene expressions for the glucose transporter, glycolytic enzymes, and lactate dehydrogenase [86-89]. Enhanced activities of hexokinase and, hence, increased glucose uptake have been observed in melanoma [65, 90]. Melanoma have demonstrated reduced numbers of flavoprotein enzymes of the electron transport chain [73]. Enhanced glycolysis and reduced activities of TCA cycle enzymes are associated with melanoma progression [91, 92].

As mentioned previously, in anaerobic metabolism, two NAD-linked dehydrogenases have binding sites for NADH. In aerobic metabolism, eight NAD-linked dehydrogenases have binding sites for NADH. Thus, a shift in the energy metabolism of cancer cells from aerobic respiration to aerobic glycolysis may reduce the interaction of NADH with NAD-linked dehydrogenases. The mitochondrial matrix space contains a high protein concentration (greater than 30%) and thus reduces the mobility of large molecules or even those with a size as small as NADH [43]. Since the interactions of the TCA cycle enzymes with each other and with the mitochondrial inner membranes further reduce the
“freedom” of movement of NADH, the metabolic shift of cancer cells may further yield an environment much less stringent for NADH motion.

Fluorescence anisotropy is useful for studying the rotational motion of a fluorescent molecule. Factors, such as protein binding and viscosity, which alter the rotational motion will also alter the fluorescence anisotropy value [93]. Thus, it may be possible to detect changes in the binding and microenvironment of NADH in cancer cells using fluorescence anisotropy measurements.

### 4.1.3 Cell NADH Fluorescence Modified by the Presence of Rotenone

To test the feasibility of using fluorescence anisotropy measurements to detect changes in NADH during metabolic perturbation, rotenone was employed to block mitochondria-associated energy metabolism in living cells. Rotenone is a well-known inhibitor of the complex I of the electron transport chain [47]. Rotenone blocks the transfer of electrons from the high-potential iron-sulfur cluster to ubiquinone [47]. As a result, NADH accumulates in mitochondria, leading to an increase in NADH fluorescence intensity [5, 12, 20, 22]. An increased ratio of free to protein-bound NADH has been reported in rotenone-treated fibroblasts [22]. Fluorescence lifetime measurements have shown that rotenone-treated endothelial cells have a decreased fluorescence lifetime as compared to the untreated control cells, suggesting an accumulation of free NADH within the mitochondria [20]. Therefore, rotenone-treated cells are expected to have higher fluorescence emission and lower fluorescence anisotropy as compared to untreated control cells.
4.1.4 Cell Suspension as an in vitro Model System to Study Cell Metabolism

Because the native NADH fluorescence of living cells is very weak, settled cell suspensions were used to generate fluorescence emissions. Cell suspensions are important metabolic in vitro systems because of preserved integrated cell metabolism [94]. However, cell suspensions can be used only for a period of four to six hours due to their limited survival time period [94]. In optical measurements, absorption and background fluorescence generated from cell culture media may be a concern. Resuspension of cells in saline solutions, such as phosphate buffered saline (PBS), can get around of these problems without modifying cellular NADH fluorescence [95]. Cell suspensions in saline solutions have been widely studied with their native fluorescence properties [7, 8, 12, 18, 23, 31, 32, 96-98].

Because different scattering and absorption may be associated with the cell suspensions of each cell type, the absorption and scattering properties of the cell suspensions were studied with reflectance spectroscopy. Changes of the reflectance spectra can be used to measure the changes of absorption and the calculated degree of linear polarization (DLP) can be used to compare the scattering of the cell suspensions. Both polarized and unpolarized reflectance measurements were used to account for the changes of fluorescence anisotropy due to the different absorption and scattering of the cell suspensions.

4.2 Materials and Methods

The experimental set-up for fluorescence and reflectance spectroscopy is the same as described in Chapter 3. The polarization orientation of the illumination light was set as
parallel to the scattering plane. A fluorescence microscopic imaging system was used to
determine the distribution of fluorescence in the cells used in this study. Melanocytes and
melanoma cells were cultured, harvested, and made into cell suspensions. To induce a
metabolic change in cells, cell suspensions were treated with rotenone. Fluorescence
spectroscopy was used to study the effects of rotenone treatment on NADH fluorescence
emission and anisotropy in the treated cells. Fluorescence and reflectance spectroscopy
was used to address the scattering and absorption properties of melanocyte and melanoma
cell suspensions and to study the fluorescence anisotropy of the melanocytes and
melanoma cells.

4.2.1 Digital Imaging System

Since UVA excitation has been observed to elicit nuclear fluorescence in some cancer
cells [23], fluorescence imaging of the monolayer cultures of melanocytes and melanoma
cells was performed to verify that cell fluorescence originated in the cytoplasm of the
cells in this study. The fluorescence imaging set-up is shown in Figure 4.5. An Olympus
IMT2 inverted microscope was used with an Olympus CoolSnap CCD camera to acquire
images. A 100W mercury arc lamp (Osram) was used as a light source for fluorescence
imaging. The excitation light at 360nm was selected by an excitation bandpass filter
(360nm±20nm, Chroma Technology) within the excitation filter wheel, and fluorescence
emissions at 465nm was collected through an emission bandpass filter (465nm±40nm,
Chroma Technology) within the emission filter wheel. A dichroic mirror (390nm) was
used to reflect the excitation light to a sample and pass the emission light from the sample
to the CCD camera. A 10x UV objective was used for image collection. Fluorescence and
phase contrast images of the cell monolayer cultures were acquired using Metamorph software (Universal Imaging Corporation).

Figure 4.5 Schematic diagram of the digital imaging system. The dashed box represents the boundary of the microscope. The dichroic mirror is a 390nm longpass filter; longer wavelengths are passed and shorter wavelengths are reflected.

4.2.2 Cell Culture

The human melanocyte and melanoma cells under investigation are listed in Table 4.3. Human melanocyte cell strain HEMn_LP (Cascade Biologics, Portland, OR), isolated from neonatal foreskin with light pigmentation, was cultured with Medium 154 (Cascade Biologics, Portland, OR). Human melanoma cell lines WM793 and WM115 (The Wistar Institute, Philadelphia, PA) were cultured with Dulbecco’s modified Eagle’s
medium (DMEM, ICN Biomedical Inc, Aurora, OH) supplemented with 10% fetal bovine serum (Gibco) and 5μg/ml insulin (Sigma). Both WM793 and WM115 are vertical growth phase melanoma. All of the cells were routinely cultured in a 37°C, humidified cell culture incubator (NAPCO) with 5% CO₂ and 95% air. For fluorescence study, human melanocytes with passage number of 3 to 9, WM115 melanoma cells with passage number of 70 to 77, and WM793 melanoma cells with passage number of 69 to 75 were used.

Table 4.3 The melanocytes and melanoma cell lines under investigation in this study

<table>
<thead>
<tr>
<th>Cell</th>
<th>Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocyte</td>
<td>Primary cell</td>
<td>Cascade Biologics</td>
</tr>
<tr>
<td>WM793</td>
<td>VGP melanoma</td>
<td>Wistar Institute</td>
</tr>
<tr>
<td>WM115</td>
<td>VGP melanoma</td>
<td>Wistar Institute</td>
</tr>
</tbody>
</table>

For spectra collection, cultured cells were trypsinized by incubating the cells with trypsin/EDTA solution (Sigma, 0.025% trypsin/0.01% EDTA for the melanocytes, and 0.25% trypsin/0.02% EDTA for the melanoma cells) when they reached near or 100% confluence. The cells were washed three times with phosphate buffered saline (PBS) to remove any residual medium. The fluorescence of the third wash was measured to account for any background fluorescence generated by the residual media. Cell concentrations were measured with a hemocytometer and cell viabilities were determined by trypan blue exclusion assay.

4.2.3 Rotenone Treatment

To investigate the effect of rotenone treatment on the fluorescence anisotropy, the melanocytes and melanoma cells were resuspended in their culture media after
trypsinization. The cell concentrations of the suspensions used were $8 \times 10^6$ to $1 \times 10^7$ cells/ml for the melanocytes, $8 \times 10^6$ cells/ml for WM115, and $1 \times 10^7$ cells/ml for WM793. For rotenone treatment, 1 ml of cell suspension was split evenly into two sterile 1.5 ml centrifuge tubes. Cells were allowed to settle for 2 to 3 minutes, and 2.5μl of supernatant was removed from each tube. A 2.5μl aliquot of 1mM rotenone (Sigma-Aldrich) stock solution was added and mixed with the cell suspension in one tube to make a final concentration of 5μM rotenone. Because the rotenone stock solution was made by dissolving rotenone in ethanol, 2.5μl of ethanol was added and mixed with the cell suspension in the other tube as a control. The cell suspensions were incubated for 10 minutes at room temperature and gently swirled to prevent cell precipitation. Following the incubation, the cells were spun down, washed three times with PBS, and finally resuspended in 0.5ml PBS in each tube for fluorescence spectral measurements. Cell viabilities were checked before the rotenone treatment and after the fluorescence spectral measurements.

4.2.4 Fluorescence and Reflectance Spectroscopy of Cell Suspension

Due to the different scattering and absorption properties of the cell suspensions, the melanocytes and melanoma cells were resuspended in PBS at different concentrations ranging from $5 \times 10^6$ to $6 \times 10^7$ cells/ml. Polarized fluorescence and polarized reflectance spectra were then collected for each cell suspension. Cell viabilities were checked before and after all of the fluorescence and reflectance measurements were done. For the spectral measurement of each cell suspension, 0.2ml of cell suspension was transferred into a 0.2ml custom-made mini-petri dish with an inner diameter of 1.302cm. After
waiting for 5 minutes to allow the cells to settle, polarized fluorescence spectra were collected as described in Chapter 2. Briefly, sequential polarized fluorescence spectra were collected with the polarization orientation between the polarizer and analyzer set as parallel, perpendicular, perpendicular, and parallel. The two parallel polarized fluorescence spectra were compared to determine if photobleaching occurred. Immediately following the collection of the polarized fluorescence spectra, the light source was switched from the mercury light to the halogen light, and polarized reflectance spectra were collected in the same manner. Conventional non-polarized reflectance spectra were then collected by removing the polarizers from the light path. The non-polarized reflectance spectra were used to study the variation in absorption of the cell suspensions as a function of cell type and concentration.

Polarized fluorescence and polarized reflectance spectra were imported into Matlab and the fluorescence anisotropy (FA) and degree of linear polarization (DLP) were calculated using Equation 1.3 and Equation 1.2, respectively. The FA and DLP were calculated for each cell suspension using the methods described in Chapters 2 and 3. Briefly, to calculate FA for each cell suspension, the two parallel and two perpendicular polarized fluorescence spectra collected were averaged, respectively. FA was then calculated with the averaged spectra. FA was smoothed with a median filter, if needed, to suppress spike noise caused by the spectroscopic measurement system. The DLP for each cell suspension was calculated in a similar manner using the averaged polarized reflectance spectra.
4.3 Results

The fluorescence images of cell monolayer cultures are presented, showing that the fluorescence of the cells was generated from the cytoplasm of the cells. The rotenone treatment of the cells resulted in an increase in fluorescence emission and a decrease in fluorescence anisotropy of the cells. The experimental results of cell suspensions with reflectance spectroscopy have shown different scattering and absorption properties with the cell suspensions of different cell types at different cell concentrations. The effects of the scattering and absorption of the cell suspensions on fluorescence anisotropy measurements were measured. The fluorescence anisotropies of the melanocytes and melanoma cells were compared after accounting for the scattering and absorption of the cell suspensions.

4.3.1 Fluorescence Imaging of Cell Monolayer Culture

Cell monolayer cultures, which were grown in two-chambered coverglasses (Lab-Tek), were used for fluorescence imaging. Because both the melanocytes and melanoma cells tended to detach from the coverglass when washed and maintained in PBS, fluorescence images were taken when the cells were maintained in media. Fluorescence images were taken with excitation at 360nm and emission at 465nm. Phase contrast and fluorescence images of the melanocytes and melanoma cells are shown in Figure 4.6. The melanocytes appear bipolar, tripolar, or multipolar in the phase contrast image. Some WM115 cells appear extraordinarily big and very flat, while most WM115 cells have a spindle-like or round shape. WM793 cells are epithelioid, and all WM793 cells are flat. The fluorescence images of the three cell types demonstrate that cell fluorescence is
Figure 4.6 Phase contrast (left) and fluorescence (right) images of human melanocytes (top), WM115 melanoma cells (middle), and WM793 melanoma cells (bottom). Fluorescence images were acquired with excitation at 360nm and emission at 465nm. Magnification was 10⁻×.
located within the cytoplasm of the cells. The nuclei of the cells are non-fluorescent. In some cases, diffuse fluorescence can be seen from some nuclei due to the superimposed cytoplasm above the nuclei.

### 4.3.2 Effect of Rotenone Treatment on the Fluorescence Emission and Anisotropy

Fluorescence spectra with polarized excitation at 360nm were collected from melanocytes, WM115, and WM793 cell suspensions on different days. The fluorescence anisotropy of a given cell suspension was calculated as described in the methods; the fluorescence anisotropies for like specimens collected on different days were then averaged. For rotenone treated cells, viabilities were calculated before rotenone treatment and after fluorescence measurements and are summarized in Table 4.4. Cell viabilities were always above 90% during the experiments. No background fluorescence was seen in spectra collected from the PBS saved after the third wash of the cell suspensions. No photobleaching was observed by comparing the two parallel polarized fluorescence spectra as described in the methods.

Since fluorescence spectra were not collected without an analyzer in place, the fluorescence spectrum for each cell suspension was calculated by adding the averaged parallel and perpendicular fluorescence spectra. After rotenone treatment, cell fluorescence from all cell types increased appreciably relative to the control cells, as shown in Figure 4.7. The total fluorescence, $F_T$, was calculated for each spectrum by integrating the fluorescence intensity over the wavelength range from 430nm to 550nm. This wavelength range was selected as being emissions primarily resulting from NADH fluorescence. The change in the fluorescence due to rotenone treatment, $\Delta F_T$, was then
calculated as the difference between the total fluorescence of the rotenone-treated cells and the control cells divided by the total fluorescence of the control cells, or:

$$\Delta F_T = \frac{\int_{430nm}^{550nm} F.I_{RT} d\lambda - \int_{430nm}^{550nm} F.I_C d\lambda}{\int_{430nm}^{550nm} F.I_C d\lambda}$$

The results of the effect of rotenone on the total fluorescence are summarized in Table 4.5. The average increase in fluorescence intensity is 34.9-43%. When comparing the average increase in fluorescence between any of the cell types, no significant difference is seen.

Table 4.4 Cell viabilities before the rotenone treatment and after the collection of fluorescence spectra.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Before rotenone treatment (%)</th>
<th>After fluorescence measurement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocyte</td>
<td>96.8±4.5 (n=2)</td>
<td>95.5±2.8 (n=2)</td>
</tr>
<tr>
<td>WM115</td>
<td>96.9±0.3 (n=4)</td>
<td>95.0±0.5 (n=4)</td>
</tr>
<tr>
<td>WM793</td>
<td>97.5±1.8 (n=4)</td>
<td>95.6±3.5 (n=4)</td>
</tr>
</tbody>
</table>

Figure 4.7 Representative fluorescence spectra of the melanocytes (left), WM115 (middle), and WM793 (right) with polarized excitation at 360nm. The cell concentrations were $8 \times 10^6$ cells/ml for both the melanocytes and WM115 cells and $1 \times 10^7$ cells/ml for WM793 cells. For each cell suspension, the fluorescence spectrum was obtained by adding the averaged parallel polarized fluorescence
spectrum and the averaged perpendicular polarized fluorescence spectrum. The upper spectra (in blue solid) were taken of the cells with rotenone treatment. The lower spectra (in magenta dashed) were taken of the control cells. Integration times were 1.5 seconds for the melanocytes, 0.8 seconds for WM115, and 1 second for WM793 cells.

Table 4.5 Change in fluorescence intensity for the different cell types after rotenone treatment. Fluorescence was exited at 360nm. No significant difference in the change is seen between any of the cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Percent change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocyte</td>
<td>43.0±19.3 (n=2)</td>
</tr>
<tr>
<td>WM115</td>
<td>34.9±14.3 (n=4)</td>
</tr>
<tr>
<td>WM793</td>
<td>36.3±22.8 (n=4)</td>
</tr>
</tbody>
</table>

The fluorescence anisotropies for each cell type were calculated with and without rotenone treatment. The fluorescence anisotropy was lower for the rotenone-treated cells than for the control cells in all three cell types tested. The average values of the fluorescence anisotropies for the three cell types are shown in Figure 4.8. The WM793 cells showed a smaller decrease in fluorescence anisotropy than the melanocytes and WM115 cells.

Figure 4.8 Fluorescence anisotropy of melanocyte (left), WM115 (middle), and WM793 (right) cell suspensions. The cell concentrations were between $8\times10^6$ to $1\times10^7$ cells/ml for the melanocytes, $8\times10^6$ cells/ml for WM115 cells, and $1\times10^7$ cells/ml for WM793 cells. The mean values of fluorescence anisotropy were calculated from 2 batches of melanocytes and 4 batches of WM115 and WM793
cells, respectively. Fluorescence anisotropies decreased after rotenone treatment (blue solid) as compared to those of the control cells (magenta dashed) for the three cell types.

4.3.3 Reflectance Spectra of the Cell Suspensions

The levels of pigmentation in the melanocytes and melanoma cells used in this study varied widely. As a result, the absorption properties also varied widely. Conventional non-polarized reflectance spectra were collected from the settled melanocyte and melanoma cell suspensions at concentrations from $5 \times 10^6$ to $6 \times 10^7$ cells/ml. The reflectance spectra for a given cell type and concentration were averaged over the measurements of different batches of cells on different days. Each average spectrum was then normalized against the maximum intensity of the average spectrum. The normalized reflectance spectra for each cell type are shown in Figure 4.9. All three cell types show a stronger absorption at higher cell concentrations, which leads to a red-shift of the reflectance spectra. The absorption effect was much more pronounced for the melanocytes than for the two melanoma cell lines.

Figure 4.9 Reflectance spectra of melanocyte (left), WM115 (middle), and WM793 (right) cell suspensions at different cell concentrations. The reflectance spectra were averaged over the measurements on different days and normalized against the maximum intensities of the averaged
spectra. The cell concentrations (cells/ml) and numbers of baths of cell cultures are shown in the legends.

Table 4.6 Effect of cell concentration on the peak wavelength (in nm)* of the reflectance spectra of the settled cell suspensions.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Concentration 6×10⁷cells/ml</th>
<th>2×10⁷cells/ml</th>
<th>1×10⁷cells/ml</th>
<th>7×10⁶cells/ml</th>
<th>5×10⁶cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocyte</td>
<td>680.8±9.1(n=4)</td>
<td>640.1±20.2(n=3)</td>
<td>615.6±8.7(n=6)</td>
<td>NA</td>
<td>613.0±7.3(n=6)</td>
</tr>
<tr>
<td>WM115</td>
<td>622.9±3.8(n=4)</td>
<td>614.5±4.0(n=6)</td>
<td>606.0±6.1(n=6)</td>
<td>606.1±4.9(n=6)</td>
<td></td>
</tr>
<tr>
<td>WM793</td>
<td>621.9±4.3(n=4)</td>
<td>610.2±4.6(n=4)</td>
<td>606.3±5.3(n=5)</td>
<td>NA</td>
<td>607.6±5.5(n=5)</td>
</tr>
</tbody>
</table>

* Mean ± SD (nm). Statistics: Student’s t-test.
a P<0.0001 relative to WM115 and WM793 at a concentration of 6×10⁷cells/ml; P<0.05 relative to the melanocytes at a concentration of 2×10⁷cells/ml;
b P<0.05 relative to WM115 and WM793 at a concentration of 2×10⁷cells/ml; P<0.05 relative to the melanocytes at a concentration of 1×10⁷cells/ml;
c P<0.05 relative to WM115 at a concentration of 2×10⁷cells/ml;
d P<0.05 relative to WM115 at a concentration of 7×10⁶cells/ml;
e P<0.05 relative to WM793 at a concentration of 2×10⁷cells/ml;

The wavelength where the reflectance spectra was maximum, or the peak wavelength, was also averaged over different spectra collected for a given cell type and concentration. The values of peak wavelength are summarized in Table 4.6. The peak wavelength was red-shifted with increasing concentration of the cell suspensions for each cell type. All three cell types showed a significant red-shift of the peak wavelength at the highest concentrations as the cell concentration increased from 2×10⁷cells/ml to 6×10⁷cells/ml (P<0.05). The melanocytes also showed a significant red-shift between 1×10⁷cells/ml and 2×10⁷cells/ml (P<0.05). However, no statistically significant shift in peak wavelength was observed for the lowest cell concentrations from 5×10⁶cells/ml to 1×10⁷cells/ml. The lack of red-shift at the lowest concentrations suggests that absorption is considerably reduced.

No statistical difference was seen between the peak wavelengths of WM115 and WM793 at any concentration, suggesting similar absorption by these two melanoma cell
lines. However, the peak wavelength of the melanocytes was significantly higher than either of the two melanoma cell lines at the highest two concentrations of $6 \times 10^7$ cells/ml ($P < 0.0001$) and $2 \times 10^7$ cells/ml ($P < 0.05$), suggesting a much stronger absorption by the melanocytes than the melanoma cells at these high cell concentrations. There was no statistically significant difference of the peak wavelength between the melanocytes and melanoma cells at the concentrations of $1 \times 10^7$ cells/ml and $5 \times 10^6$ cells/ml, suggesting that the melanocytes and melanoma cells shared a similar absorption at these low cell concentrations.

4.3.4 Polarized Reflectance Spectroscopy of the Cell Suspensions

To compare the optical scattering of the melanocyte and melanoma cell suspensions at different concentrations, polarized reflectance spectra were collected immediately following the collection of polarized fluorescence spectra of the settled cell suspensions at concentrations ranging from $7 \times 10^6$ to $6 \times 10^7$ cells/ml. Representative polarized reflectance spectra for each of the three cell types are shown in Figure 4.10. The reflectance spectra of the melanocytes are much lower than those of the two melanoma cell lines, suggesting much stronger absorption of the melanocytes than the two melanoma cell lines at the concentration of $2 \times 10^7$ cells/ml. Since DLP can be used to study multiple scattering within turbid media, where stronger scattering depolarizes incident polarized light to a greater extent and results in a lower DLP, the DLP was calculated as shown in Equation 1.2 by first averaging the two spectra collected at the parallel and perpendicular orientations of the polarizers, respectively. The average DLP was calculated by averaging the DLP calculated from different batches of cells of the
same type and concentration collected on different days. As shown in Figure 4.11, the DLP increased as the cell concentration decreased for the three cell types, meaning that as the cell layer decreased in thickness, the depolarization of the incident light decreased.

Figure 4.10 Polarized reflectance spectra of the melanocyte (left), WM115 (middle), and WM793 (right) cell suspensions at the concentration of $2\times10^7$ cells/ml. Integration times were 0.5 seconds for the melanocytes, 0.35 seconds for WM115, and 0.4 seconds for WM793 cells.

Figure 4.11 The average DLP of the backscattered light from the melanocyte (left), WM115 (middle), and WM793 (right) cell suspensions with different cell concentrations. Average DLP was calculated from the measurements on different batches of cells. The cell concentrations (cells/ml) and numbers of batches of cells are shown in the legends.
Figure 4.12 Comparison of DLP between different cell types at the same cell concentrations (A-C). DLP spectra were averaged over the measurements of different batches of cells. (A) DLP for the cell suspensions of $6 \times 10^7$ cells/ml. (B) DLP for the cell suspensions of $2 \times 10^7$ cells/ml. (C) DLP for the cell suspensions of $1 \times 10^7$ cells/ml. (D) DLP for the cell suspensions of $1 \times 10^7$ cells/ml for the melanocytes and WM793 cells and $7 \times 10^6$ cells/ml for WM115 cells. Dashed lines represent +/- one standard deviation for the data set.

When comparing the values of DLP of the three cell types at the same cell concentrations, the data clearly show differences in the optical scattering properties. As shown in Figure 4.12, the DLP of the melanocytes was higher than the DLP of the two melanoma cells at the two highest concentrations (in Figure 4.12A, B). These are the same concentrations where a statistically significant difference in the peak wavelengths due to absorption was observed in the reflectance spectra. Thus, the statistically significant increase of the absorption by the melanocytes at high cell concentrations...
results in a higher value of DLP for the melanocytes than for the melanoma cells. Similar results were reported in the literature [99, 100], where the authors suggest that absorption results in a higher DLP due to the absorption of the highly scattered photons with long optical pathlengths, thus eliminating their contribution to the backscattered light.

At low cell concentrations (<1×10⁷ cells/ml), absorption by the settled cells was relatively weak, and the absorption of the melanocytes and the two melanoma cells was not statistically different. When comparing the cells with similar absorption, such as the WM115 and WM793 cells at equal concentration, the DLP is not the same. This suggests that the difference in DLP is not associated with absorption, but with differences in scattering. Therefore, the difference in DLP seen between the melanocytes and melanoma cells when their absorptions are equivalent cannot be attributed to absorption.

The DLP of the WM115 cells was always lower than that of WM793 cells at the same cell concentrations, suggesting a stronger scattering of WM115 cells than WM793 cells. At the highest cell concentrations where absorption was significant, the DLP of the melanocytes was higher than the two melanoma cell lines (in Figure 4.12A, B). However, as the concentration was reduced to 1×10⁷ cells/ml, the DLP of the melanocytes (Figure 4.12C) was between that of the two melanoma cell lines, suggesting that absorption no longer played a significant role in the DLP. The DLP of the melanocytes at the cell concentration of 1×10⁷ cells/ml was practically the same as that of WM115 cells at of 7×10⁶ cells/ml (Figure 4.12D), suggesting that the same scattering property could be obtained by lowering the cell concentration for WM115 cells.

4.3.5 Polarized fluorescence Spectroscopy of the Cell Suspensions
Polarized fluorescence spectra were collected from the three cell types at different cell concentrations. Cell viabilities were checked before and after each measurement, and are summarized in Table 4.7. The cell viabilities for the three cell types were always above 90%. When comparing the two parallel polarized fluorescence spectra, which were the first and fourth spectra collected, no photobleaching was seen. Representative polarized fluorescence spectra of the three cell types are shown in Figure 4.13.

Table 4.7 Cell viabilities before and after fluorescence and reflectance measurements of the three cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Before measurement</th>
<th>After measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocyte</td>
<td>94.6±3.6 (n=6)</td>
<td>90.8±4.1 (n=6)</td>
</tr>
<tr>
<td>WM115</td>
<td>91.8±4.0 (n=6)</td>
<td>88.5±5.1 (n=6)</td>
</tr>
<tr>
<td>WM793</td>
<td>93.9±4.6 (n=6)</td>
<td>89.8±4.9 (n=6)</td>
</tr>
</tbody>
</table>

Figure 4.13 Polarized fluorescence spectra of the melanocyte (left), WM115 (middle), and WM793 (right) cell suspensions at the concentration of 2×10^7 cells/ml. Integration times were 1.5 seconds for the melanocytes, 0.5 seconds for WM115, and 1 second for WM793 cells.
Figure 4.14 Comparison of the fluorescence anisotropy of the cell suspensions with different cell concentrations (A-C) and between different cell types (D). Fluorescence anisotropy spectra were averaged over measurements of different batches of cells. (A) Fluorescence anisotropy of melanocyte suspensions with cell concentration from $1 \times 10^7$ to $6 \times 10^7$ cells/ml. (B) Fluorescence anisotropy of WM115 cell suspensions with cell concentration from $7 \times 10^6$ to $6 \times 10^7$ cells/ml. (C) Fluorescence anisotropy of WM793 cell suspensions with cell concentration from $1 \times 10^7$ to $6 \times 10^7$ cells/ml. (D) Comparison of fluorescence anisotropy between the melanocytes ($1 \times 10^7$ cells/ml), WM115 cells ($7 \times 10^6$ cells/ml), and WM793 cells ($1 \times 10^7$ cells/ml). Dashed lines represent +/- one standard deviation for the data set.

Fluorescence anisotropy was calculated first by averaging the parallel and perpendicular spectra collected on each cell type and concentration, respectively. The average fluorescence anisotropy was then calculated by averaging the fluorescence anisotropy for a cell type and concentration over different batches collected on different days. As shown in Figure 4.14(A-C), fluorescence anisotropy, like DLP described above,
increased as the cell concentration decreased for the three cell types, suggesting a dependence of fluorescence anisotropy on the scattering of the cell suspensions. The behavior of the fluorescence anisotropy of the melanocytes was similar to the DLP; the fluorescence anisotropy was remarkably higher than for the two melanoma cell lines at high cell concentrations (>2×10^7 cell/ml). This difference could partly be due to the much stronger absorption of the melanocytes relative to the melanoma cells at the high cell concentrations. However, the fluorescence anisotropy of the melanocytes was still appreciably higher than the fluorescence anisotropy of the two melanoma cell lines at the lowest cell concentrations (Figure 4.14D), whereas the two melanoma cell lines showed similar fluorescence anisotropy. The results with the polarized reflectance measurements showed that the melanocytes had equal to higher scattering than the two melanoma cell lines at these cell concentrations. Therefore, the higher fluorescence anisotropy for the melanocytes was not due to the difference in scattering and/or absorption properties of the cell suspensions which favors a higher value of fluorescence anisotropy for the melanocytes. As a result, the higher value of fluorescence anisotropy for the melanocytes could reflect more binding of NADH and/or a more stringent microenvironment for NADH in the melanocytes than in the melanoma cells.

4.3.6 Comparison of Fluorescence Intensity Ratio of Melanocytes and Melanoma Cells

Fluorescence emission of the protein-bound NADH is blue-shifted as compared to that of the free NADH. It was suggested in the literature that the ratio of fluorescence intensity at 410 and 465nm could provide a sensitive test of NADH binding [12].
Fluorescence and reflectance spectra with the polarization configuration described previously were collected from the cell suspensions of the melanocytes and the two melanoma cell lines. The cell concentration was $1 \times 10^7$ cells/ml for the melanocytes and WM793 cells and $7 \times 10^6$ cells/ml for WM115 cells. Normalization of the fluorescence spectra was experimentally shown to recover intrinsic fluorescence of fluorophores from turbid media [101]. Using the method described, the normalized fluorescence ratio spectra (FRS) for each cell type were calculated as

$$ FRS = \frac{I_{f_{\parallel}} - I_{f_{\perp}}}{I_{r_{\parallel}} - I_{r_{\perp}}} $$

where terms are the same as defined for FA and DLP ($f$ denotes fluorescence, and $r$ denotes reflectance). The FRS was averaged over 6 measurements on 6 batches of cells for each cell type. Each average FRS was then normalized to its peak emission as shown in Figure 4.15 to compare the different cell types. The peak emissions from the average FRS were around 420nm. However, the normalized fluorescence ratio spectrum of the melanocytes shows an appreciable blue-shift as compared to those of the two melanoma cells.

Due to an artifact in our data at 410nm which was caused by the mini-petri dish, the ratio of fluorescence intensity at 410nm to 465nm could not be computed, so the ratio of 430 nm to 465 nm was calculated instead. The ratios of fluorescence intensity at 430 and 465nm for the melanocytes and melanoma cells are shown in Table 4.8. The fluorescence intensity at each wavelength (430 or 465nm) was obtained by integrating the fluorescence ratio spectra over a wavelength range of $\pm 1$ nm about the mean wavelength value. The fluorescence intensity ratio was averaged over 6 measurements on 6 batches of cells for
Figure 4.15 Fluorescence ratio spectra of the different cell types. Fluorescence ratio spectra were averaged over 6 measurements of 6 batches of cells for each of the three cell types, and normalized against the maximum emission intensities.

Table 4.8 Fluorescence intensity ratio* at 430 and 465nm for the different cell types.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Ratio of fluorescence intensity at 430 and 465nm (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocyte</td>
<td>1.844±0.095†</td>
</tr>
<tr>
<td>WM115</td>
<td>1.750±0.019‡</td>
</tr>
<tr>
<td>WM793</td>
<td>1.733±0.016</td>
</tr>
</tbody>
</table>

* Mean ± SD, n=6 from 6 independent experiments. Statistics: Student’s t-test.
† p<0.05 relative to WM793 and WM115;
‡ p=0.13 relative to WM793.

Each of the three cell types. As seen in Table 4.8, the melanocytes display a significantly higher ratio of fluorescence intensity than the two melanoma cell lines (p<0.05). No statistical difference was seen in the ratio of the fluorescence intensities for the two melanoma cell lines. The higher ratio of fluorescence intensity at 430 and 465nm for the melanocytes relative to the melanoma cells suggests that NADH binding was higher in the melanocytes than in the melanoma cells. The higher ratio of 430 to 465 nm
fluorescence intensity is consistent with the higher fluorescence anisotropy calculated in the melanocytes relative to the melanoma cells.

**4.4 Discussion**

Based on the assumption that enhanced glycolysis and reduced aerobic respiration of cancer cells may change the physicochemical properties of NADH, fluorescence anisotropy of the cellular NADH of the melanocytes and melanoma cells were collected to probe the different metabolic properties of the cells. To evaluate the feasibility of the fluorescence anisotropy measurement of cellular NADH for tracking metabolic changes in living cells during metabolic perturbation, rotenone treatment was applied to induce metabolic changes of the cells. Rotenone inhibits the complex I of the electron transport chain [47], and thus, blocks the transfer of electrons through the electron transport chain, which in turn arrests the oxidation of NADH at the site of the complex I. Therefore, NADH accumulates in the mitochondria with rotenone present. Studies have demonstrated an increase of NADH fluorescence intensity after rotenone inhibition in various types of cells [5, 12, 20, 22]. In addition, spectral fitting analysis of the fluorescence spectra of rotenone-treated fibroblasts has revealed an increased free/bound NADH ratio as compared to the untreated cells [22]. Fluorescence lifetime imaging has also demonstrated a decreased fluorescence lifetime for endothelial cells treated with rotenone compared to control cells, which was attributed to the accumulation of free NADH within or close to the mitochondria [20]. A recent study has shown that free NADH constitutes about 60% of the total matrix NADH in intact mitochondria, and the rest of the pool of NADH is protein-bound and contributes to about 80% of the
fluorescence emission [21]. As the total matrix NADH level increases, the pool of the protein-bound NADH saturates, whereas the pool of free NADH increases linearly as the total NADH level increases [21]. The mitochondrial NADH level increases with rotenone inhibition of the electron transport chain. Thus, the protein-bound NADH pool in the mitochondria could saturate with the increase of NADH level induced by rotenone inhibition, while the free NADH pool in the mitochondria could still linearly increase. Therefore, the relative increase in the free NADH pool could account for the decreased fluorescence anisotropy of the melanocytes and melanoma cells seen in our experiments with rotenone treatment. The effect of the rotenone inhibition on cellular NADH fluorescence also demonstrates that oxidative phosphorylation through the electron transport chain was active in all of the three cell types. However, it does not necessarily mean that the two melanoma cells have normal aerobic energy metabolism, such as a normal activity of the TCA cycle and tight coupling of glycolysis and respiration. In fact, studies have indicated both inactivated glycerol-phosphate and malate-aspartate shuttles [102, 103], while others have shown enhanced malate-aspartate shuttle [64] or glycerol-phosphate shuttle [71] in cancer cells. Therefore, the activity of the electron transport chain of the two melanoma cells could be maintained by shuttling of the glycolytically produced NADH from the cytosol into the mitochondria.

In order to compare fluorescence anisotropy data between the different types of cells, differences in optical properties of the cell suspensions, namely absorption and scattering, need to be addressed. Reflectance spectroscopy was used to compare the different optical properties of the melanocyte and melanoma cell suspensions. The melanocytes showed much stronger absorption than the melanoma cells at high cell concentrations (> \(2 \times 10^7\))
cell/ml) as indicated by the red-shift of the reflectance spectra, resulting in a higher degree of linear polarization and fluorescence anisotropy for the melanocytes. The effect of absorption on DLP and fluorescence anisotropy may be caused by the loss (absorption) of highly scattered long-pathlength photons in the backscattered light [99, 100]. The difference of absorption between the melanocytes and the melanoma cells was minimized with dilution of the cell suspensions, and the relatively weak absorption at the lowest cell concentrations might be eliminated through normalization. WM115 cells showed the strongest scattering property and WM793 cells showed the weakest scattering property among the three types of cells. The different scattering properties may be caused by the different level and shape of the index-mismatched structures in these cells.

Fluorescence anisotropy measurements revealed appreciably higher fluorescence anisotropy with the melanocytes than the melanoma cells. This difference in fluorescence anisotropy was not caused by a difference in optical properties of the cell suspensions favoring higher fluorescence anisotropy for the melanocytes. Thus, this higher fluorescence anisotropy suggests a higher protein-bound NADH level and/or more stringent microenvironment for NADH in the melanocytes. The ratio of fluorescence intensity at 430 and 465nm also supports high levels of binding of NADH in the melanocytes. As described in the introduction, two NADH binding sites exist on NAD-linked dehydrogenases for anaerobic metabolism, while eight NADH binding sites exist on NAD-linked dehydrogenases for aerobic metabolism. The mitochondrial matrix also provides a more stringent environment for proteins or even small molecules like NADH than the cytosol. Thus, the metabolic shift of normal aerobic metabolism to enhanced glycolysis and reduced respiration may decrease the binding of NADH and provide a less
stringent microenvironment for NADH in the cytosol. Aerobic glycolysis is a characteristic of many tumors [48], which is accompanied with significantly enhanced activities of glycolytic enzymes, such as hexokinase [59-65], phosphofructokinase [59], glyceraldehyde-3-phosphate dehydrogenase [58, 66-68], pyruvate kinase [58, 68, 69], and lactate dehydrogenase [22, 58, 60, 61, 70, 71], and reduced activities of the enzymes of the TCA cycle and the electron transport chain, such as citrate synthase [72], α-ketoglutarate dehydrogenase [61], succinate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase [22, 60, 73, 74], cytochrome c oxidase [60, 75-79] and ATP synthase [56, 66, 68, 76, 79, 80]. In addition, the pyruvate translocator has been shown to have decreased activity in tumor mitochondria [104]. The function of adenine nucleotide translocator, another enzyme integral to the process of oxidative phosphorylation, is also reduced in cancer cells [75, 79]. Moreover, a truncated TCA cycle in cancer cells functions for biosynthesis with efflux of TCA cycle intermediates from the mitochondria to the cytosol rather than for oxidation [105, 106]. Studies with inhibitors of the electron transport chain have demonstrated that the ATP levels of cancer cells are not affected after inhibition of the electron transport chain [82, 107], whereas inhibition of glycolysis severely depletes ATP in cancer cells [108]. Fully transformed cells are most dependent on glycolysis and least dependent on the mitochondria for ATP synthesis [82], revealing an association between the progressively increased glycolytic rate and reduced respiration capacity with cancer progression [53, 67, 69, 82-84]. Enhanced glycolysis not only meets the energy demand of cancer cells, but also provides the cancer cells with an abundance of phosphometabolites for biosynthesis [60, 64, 103, 109]. Furthermore, enhanced glycolysis by proliferating cells can minimize oxidative stress in the cells during the
phases of enhanced biosynthesis and cell division [102]. Therefore, aerobic glycolysis observed in most primary and metastatic cancer cells is the result of adaptation to consistent environmental pressures (i.e. hypoxia) in pre-malignant lesions [57]. Aerobic glycolysis of cancer cells may reduce NADH binding and provide a less stringent environment for NADH in the cancer cells. Thus, enhanced glycolysis and reduced respiration result in a decrease in fluorescence anisotropy as demonstrated with the melanoma cells in this study.

Since aerobic glycolysis is constitutively upregulated in tumors, cells derived from tumors typically maintain their metabolic phenotypes in culture under normoxic conditions [57]. Thus, the data of the fluorescence anisotropy measurement of cellular NADH of cultured cells in vitro may apply to in vivo tissues. Hypoxia in tumor tissues induces anaerobic glycolysis which will provide increased contrast for the fluorescence anisotropy measurement. Aerobic glycolysis is a near universal phenomenon of cancers [48]. Progressively increased glycolytic rate and reduced respiration capacity are associated with cancer progression [53, 67, 69, 82-84]. Therefore, fluorescence anisotropy measurement of cellular NADH may potentially allow differentiation of cancer cells at different progression stages, such as occurs with melanoma.

4.5 Future Work

Cell and tissue diagnostics have been developed based on the difference in metabolism. The enhanced glycolysis in cancer cells has been targeted with extrinsic markers, such as fluorophore-labeled [110, 111] or radioactive element-labeled deoxyglucose [112-114]. Since the enhanced glycolysis and decreased aerobic respiration
of cancer cells may change the physicochemical properties of cellular NADH, fluorescence anisotropy measurement of cellular NADH binding may provide a non-invasive method for cancer cell detection. In order to further demonstrate the feasibility of this method, studies need to be carried out in two areas. One is to develop optical instrumentation which can be applied to in vivo human tissue. Since human tissue is a complex structure with complicated optical properties, fluorescence anisotropy measurements will suffer from the absorption and scattering of the tissue. Novel optical instrumentation and/or data processing techniques which can effectively get around of these problems will be promising in in vivo fluorescence anisotropy measurements. The second area is to measure the fluorescence anisotropy of other types of cancer cells. Enhanced glycolysis is a near universal phenomenon of many cancers [48]. Progressively increased glycolytic rate and reduced respiration capacity are associated with cancer progression [53, 67, 69, 82-84]. Therefore, fluorescence anisotropy measurements of cellular NADH will be expected to differentiate cancer cells of various cancer types at different progression stages. In the case of melanoma progression, the fluorescence anisotropy of melanocytic cells at various progression stages from benign nevi to metastatic melanoma should be measured. In addition, biochemical analysis of the metabolic pathways (glycolysis, TCA cycle, and oxidative phosphorylation) of the cancer cells and their normal counterparts needs to be carried out to provide a solid foundation for the interpretation of the results of the fluorescence anisotropy measurements.

Reference:


24. Croce, A.C., et al., *Autofluorescence properties of isolated rat hepatocytes under different metabolic conditions.* Photochemical & Photobiological Sciences :


Chapter 5

Enzymatic Erosion of Ex Vivo Acellular Dermis Revealed with Fluorescence and Reflectance Spectroscopy

5.1 Introduction

Degradation of extracellular matrix (ECM) is an essential step in tumor invasion and metastasis. Dermal matrix is the major source of the fluorescence and light scattering of skin. Tumor-induced degradation of the dermal matrix is expected to change the fluorescence and light scattering properties of skin. To investigate how these fluorescence and light scattering properties are changed, human acellular dermal matrix (acellular dermis) was degraded with enzyme to mimic the process of tumor invasion. Fluorescence and reflectance spectroscopy was used to probe the changes associated with the degradation of the acellular dermis.

5.1.1 Composition and Structure of Dermis

The dermis is a connective tissue composed of collagen, elastin, and ground substance (glycosaminoglycans, water, and salt). Collagen comprises about 77% of the dry weight of skin [1]. Type I collagen is the major collagen type in the dermis and makes up 80-85% of dermal collagen, and Type III collagen makes up the remainder.
Elastin constitutes less than 2% of the dry weight of skin [1]. Unlike the epidermis, the dermis is a relatively acellular tissue in which fibroblasts are the primary cell type.

![Image of skin layers](image-url)

Figure 5.1 The structure of dermis. The papillary dermis is situated directly below the epidermis and is distinguishable from the underlying reticular dermis by the differing structure of its collagen fibers. Taken from [2].

The dermis is generally divided into two layers: the superficial papillary dermis and the deeper reticular dermis (Figure 5.1). The papillary dermis is a thin layer of about the same thickness as the epidermis (about 100µm). The papillary dermis contains thin and loosely distributed collagen and elastic fibrils as well as greater proportions of the ground substance and cellular components than the reticular dermis. The collagen fibers of the papillary dermis are 0.3 to 3µm in diameter [1]. The great bulk of the dermis is the reticular dermis, which contains coarse elastic fibers and collagen bundles and less ground substance. The collagen bundles of the reticular dermis are 10 to 40µm in diameter [1]. The collagen bundles are aligned parallel to the skin’s surface, but are
arranged randomly with respect to orientation [1]. The elastic fibers are 10 to 12 nm in
diameter and interwoven among the collagen bundles [1]. Collagen fibers are relatively
evenly distributed throughout the dermal layer, whereas elastin fibers are approximately
three times more abundant in the reticular dermis than in the papillary dermis [3].

In between the epidermis and the dermis is the basement membrane. The basement
membrane functions as an important barrier to prevent epidermal cells from invading the
dermis and to regulate material transfer between the epidermis and the dermis. Type IV
collagen is a major component of the lamina densa of the basement membrane and
constitutes greater than 95% of the collagen in the basement membrane [4]. Unlike Type
I and Type III collagens, Type IV collagen does not form fibrils [4, 5]. Instead, Type IV
collagen forms layers which are covalently linked to one another, resulting in an effective
barrier/filter structure. Anchoring fibrils, which are primarily composed of Type VII
collagen, connect the lamina densa onto the papillary dermis [6].

5.1.2 Collagen Structure

As described above, collagen is a major component of the dermis, and Type I
collagen constitutes most of the dermal collagen. Type I collagen is a complex, highly
structured aggregate of collagen molecules and has many length scales. As shown in
Figure 5.2, collagen has a hierarchical structure. A collagen molecule is a long and rigid
molecule which is composed of three polypeptide chains known as \( \alpha \) chains. Each \( \alpha \)
chain has approximately 1000 amino acids and a molecular weight of 95,000 daltons [1].
The general structure of the \( \alpha \) chain can be represented by the repeating triplet \([—X—\ Y—\Gly—]_n\) where Gly represents glycine and X and Y represent amino acids other than
glycine [1]. Collagen α chains contain about 20% proline (located at the X position) and approximately 9-13% 4-hydroxyproline located at only the Y position [1]. Each α chain is coiled in a left-handed helix, and the collagen molecule consists of three α chains wrapped around each other to form a rope-like right-handed triple helix. Collagen molecules are covalently bound together to form a microfibril, a spiraling five-stranded structure with collagen molecules oriented end to end, but the adjacent molecules staggered from one to another by a distance of 680 Å. Microfibrils wrap around each other to form fibrils, which in turn wrap around other fibrils to form collagen fibers [1]. The fibrous structure of collagen endows it with optical anisotropic properties, such as birefringence [4, 7, 8].

Figure 5.2 The hierarchical structure of collagen. Three α chains wrap around each other to form the triple helix (tertiary structure) of a collagen molecule. Collagen molecules are bound together to form microfibrils (smith microfibrils). Microfibrils wrap around each other to form fibrils, which in turn wrap around other fibrils to form collagen fibers. Taken from [9].
Intermolecular cross-links of collagen fibers contribute to the stability of the collagen fibers. Collagen cross-links are formed via two routes, one based on lysine residues and the other based on hydroxylysine residues. In the skin, lysine residue-based allysine predominates [10]. Both routes of cross-linking lead to the formation of divalent cross-links, such as dehydro-hydroxylysinonorleucine and dehydro-hydroxylysinohydroxy-norleucine, capable of linking two separate α chains [1]. As the connective tissue matures, the divalent cross-links are converted into multivalent cross-links, such as histidinohydroxylysinonorleucine, and the total number of the cross-links increases [10-13]. Collagen fluorescence results from the interaction of light with its cross-links [14].

5.1.3 Melanoma Invasion of Dermis

Tumor invasion of connective tissue is a dynamic and multi-step process, and the degradation of extracellular matrix (ECM) is an essential step. Matrix degradation is rendered by the ECM-degrading enzymes of both tumor cells and adjacent normal cells [15-17]. Matrix metalloproteinases (MMPs), a group of zinc dependent protein-degrading enzymes, play an important role in the degradation of the ECM by tumors [18]. Malignant melanoma cells can express a number of MMPs, such as MMP-1 (interstitial Type I collagenase), MMP-2 (Type IV collagenase), MMP-9 (Type IV collagenase), MMP-13 (collagenase 3), and MT1-MMP (membrane type-1 MMP) [19].

The basement membrane forms a “fence” on the dermis and protects the underlying ECM against the infiltration of cells from the epithelium. Thus, melanoma breaching of the basement membrane of skin is a crucial step of metastasis and has been found to be associated with the degradation of the Type IV and Type VII collagen of the basement
membrane [20]. Elevated expression of MMP-2 and MMP-9, which are capable of
digesting Type IV and Type VII collagen, has been reported in melanoma cells [16, 19,
21-24] and has been shown to be associated with melanoma progression [21, 23, 24]. In
addition to the elevated expression of Type IV collagenases, overexpression of Type I
collagenases, such as MMP-1 and MMP-13, correlates with the invasive phenotype of
melanoma cells [16, 19, 21, 23, 25, 26]. Type I collagenases degrade Type I and Type III
collagen, which are the major components of the dermis, and thus facilitate melanoma
penetration into the dermis.

Not only do tumor cells produce MMPs, but normal stromal cells also express MMPs
[18]. Melanoma invasion is a complex process involving the interaction of the tumor cells
and the tumor stroma [16, 17, 27-32]. MMPs-1, -2, and -3 are expressed both in the
melanoma cells at the tumor periphery and in the stromal cells adjacent to the melanoma
cells [16, 31]. MMPs-2 and -9 derived from the adjacent fibroblasts and keratinocytes
facilitate the penetration of melanoma cells through the basement membranes [30].
Enhanced expression of MMP-1 by the fibroblasts surrounding melanoma contributes to
the invasion of melanoma into the dermis [28, 29, 31].

5.1.4 Native Fluorescence of Dermis

Previous investigations have demonstrated that major skin fluorophores are located in
the dermis, and collagen plays a main role in the formation of skin fluorescence [33-37].
Collagen fluorescence is generated from its cross-links [14, 38]. The fluorescence
emission peak of collagen is red-shifted as the excitation wavelength increases,
suggesting multiple fluorophores or multiple conformations of fluorescent
macromolecules in the collagen [34]. A fluorophore of dermal collagen has been isolated and identified as a collagen cross-link which is named pentosidine [39]. However, the molecular origin of the fluorescence of dermal collagen is still largely unknown [14, 34]. Elastin, a minor component of the dermis, shows the same trend of emission peak shift as the collagen with increasing excitation wavelength [14]. Elastin fluorescence is also generated from its cross-links [14]. A fluorophore of elastin is identified as a tricarboxilic triamino pyridinium derivative with excitation and emission maxima at 325/400nm [14].

Kollias et al. studied the fluorescence spectra from normal human skin in vivo and found prominent excitation and emission (X:M) pairs of 295:340nm, 360:425nm, 390:480nm, 460:525nm, and 580:620nm, with a minor pair at 340:390nm [40]. Zeng et al. also investigated the fluorescence from in vivo human skin and found excitation and emission pairs of 350±5:455±3nm, 360:460nm, 370:465nm, 380:470nm, 390:475nm, 410:490nm, 430:504nm, 450:515nm, and 470:548nm [34]. These fluorescence patterns reflect collagen fluorescence from the dermis. Furthermore, Zeng et al. found that the fluorescence spectra from lower dermis (reticular dermis) was blue-shifted by 20nm as compared to that from upper dermis (papillary dermis), suggesting different fluorophores in these two dermal layers [34]. To explore the origin of skin fluorescence, Kollias et al. studied the fluorescence of skin extractions and found two types of fluorescent collagen cross-links, pepsin digestible collagen cross-links (PDCCL) and collagenase digestible collagen cross-links (CDCCL), contributing to two of the three bands of the fluorescence excitation spectra of skin [41]. The fluorescence properties of these two collagen cross-links and elastin cross-links are summarized in Table 5.1.
Table 5.1 The principle fluorophores of dermis.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation λ (nm)</th>
<th>Emission λ (nm)</th>
<th>Signal intensity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDCCL (pentosidine)</td>
<td>335-340</td>
<td>380-390</td>
<td>Secondary</td>
<td>[39, 41-43]</td>
</tr>
<tr>
<td>CDCCL</td>
<td>365-380</td>
<td>420-440</td>
<td>Strong</td>
<td>[41-43]</td>
</tr>
<tr>
<td>Elastin cross-links</td>
<td>420</td>
<td>500</td>
<td>weak</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>325</td>
<td>400</td>
<td>NA</td>
<td>[14]</td>
</tr>
<tr>
<td>Elastin, or collagen cross-links</td>
<td>440</td>
<td>520</td>
<td>Secondary</td>
<td>[37]</td>
</tr>
</tbody>
</table>

Dermal fluorescence provides information on aging, photoaging, and collagen denaturation. Studies with sun-protected skin of various age groups have revealed a similar fluorescence pattern among the groups and an appreciably increased fluorescence intensity with aging [33], suggesting an increase of collagen cross-links with age [13]. The fluorescence pattern of the sun-protected skin demonstrates that the collagen cross-links derived from aging share a similar fluorescence profile. It suggests that no newtypes of fluorophores are generated with age [43]. The fluorescence intensities of both PDCCL and CDCCL have been reported to increase with age [42]. In other studies, however, the increase in fluorescence intensity with age has been ascribed to PDCCL, whereas CDCCL fluorescence remains unchanged during aging [41, 43-45]. In addition to collagen fluorescence, elastin fluorescence excited at 420nm also increases with age [43]. UV irradiation on skin causes a decreased intensity of dermal fluorescence which is due to the dissolution of collagen cross-links (PDCCL) by MMPs induced by prolonged UVA irradiation [33, 41, 45]. Denaturation of dermal collagen by laser heating has been characterized by the loss of collagen fluorescence [46].

Multiphoton polarization imaging of human dermis has revealed that fluorescence emission of dermal fibers is strongly dependent on the direction of the excitation
polarization but not on the orientation of the fibers [47]. It seems that the dipoles of the fluorescent cross-links within the fibers are randomly oriented and have no relation to the fiber orientation.

5.1.5 Other Optical Properties of Dermis

Dermis shows strong multiple scattering in the visible and near-infrared wavelength region. Dermal scattering is caused by the index mismatch between collagen fibers and their surrounding matrix and determines the light penetration depth in the dermis [7]. Major scatterers in the dermis are single fibrils and scattering centers formed by the interlacement of the collagen fibrils and bundles [48]. Light of longer wavelengths suffers less scattering [49]. Major absorbers in the dermis are blood-borne hemoglobin and oxyhemoglobin, with major absorption peaks at 433nm and 556nm for hemoglobin and at 414nm, 542nm, and 576nm for oxyhemoglobin [50].

The fibrous structure of collagen contributes to dermal birefringence. The refractive index is higher along the axis (slow axis) of collagen fibers than that along the cross section (fast axis) [51]. Research with second harmonic generation polarimetry (SHG polarimetry) has been performed to study the spatial distribution of the collagen fiber orientation in human dermis [52]. The collagen fibers in the reticular dermis show a nearly uniaxial orientation in a microscopic region of 15μm in diameter. However, the collagen orientation becomes randomized as the probing area is increased to a macroscopic region of 40mm × 55mm, suggesting that the collagen fibers in the reticular dermis are entangled on a macroscopic level [52]. These results are consistent with the anatomical analysis of the dermis [1]. In contrast with the collagen fibers in the reticular
dermis, the collagen fibers in the papillary dermis do not show any specific orientation, even on the microscopic scale of 15µm in diameter [52]. No SHG signal has been detected from Type IV collagen and elastin fibers [53].

5.1.6 Changes in Optical Scattering of Connective Tissue Associated with Tumor Progression

Structural changes of the connective tissue associated with tumor progression can also be revealed by the changes in tissue scattering [14]. The scattering properties of the bulk tissue are affected mainly by the collagen network [54]. Studies of cervical cancer have demonstrated that the ECM density of the low-grade squamous intraepithelial lesion (LGSIL) is lower than that of the normal tissue and is further reduced with the high-grade squamous intraepithelial lesion (HGSIL) [55]. The reduced matrix density results in a decrease in the reduced scattering coefficient of the squamous intraepithelial lesions (SILs) as compared with the non-SIL tissues [55]. Studies of esophageal dysplasia using reflectance spectroscopy have shown that the reduced scattering coefficient of Barrett’s esophagus tissue decreases gradually as the tissue progresses from nondysplastic, to low-grade, and further to high-grade dysplasia [56]. The changes of the tissue scattering coefficient reflect alterations in the architecture of the connective tissue collagen fibers. These alterations of the collagen architecture can occur before the onset of tumor invasion and could be the result of the tumor-associated enzymatic degradation of the collagen matrix [56].

Studies of melanocytic lesions have demonstrated that the collagen matrix is reduced or destroyed at the locations where tumor cells reside [57]. The residual collagen
structures in melanoma are associated with collagenase, suggesting a role of the collagenase in facilitating ECM breakdown and melanoma invasion [58]. Therefore, as collagen in the dermis surrounding the melanoma is lost, the scattering should decrease and become lower than in the normal dermis.

5.1.7 Fluorescence Spectroscopy Reveals Structural Changes of Connective Tissue Associated with Tumor Progression

Decreased tissue fluorescence has been observed to be associated with tissue malignancy in many tissue types [59]. The decrease of tissue fluorescence has been attributed to the loss of collagen fluorescence in colon [60-63], cervix [54, 55, 64-69], esophagus [67, 70], ovary [71], and skin [36, 70, 72-74], as the tissues progress towards malignancy. In the colon and cervix the loss of collagen fluorescence has been ascribed to the screening of the collagen fluorescence by the morphologic changes of the epithelium tissue, such as thickening of the epithelium and/or replacement of the stromal tissue by tumor cells [60, 63, 64]. Other studies, however, have revealed that the fluorescence of collagen in the tumor stroma is weaker than that in the normal stroma of colon, cervix, and esophagus [54, 55, 61, 62, 67]. Collagen fluorescence immediately beneath the basement membrane of dysplastic epithelium is less than that in deeper stroma [55]. In nonmelanoma skin cancer, the dermal tissue which surrounds the tumor nests yields much less fluorescence than the normal dermis [36, 73].

Collagen cross-links are major fluorophores of the connective tissue. Thus, decreased collagen fluorescence in the tumor tissue is ascribed to a reduced number of collagen cross-links in the tumor stroma, and is suggested to be the result of degradation of
collagen fibers by collagenases secreted by the tumor and stromal cells [54, 55, 67]. Decreased collagen fluorescence is associated with a decreased density of the collagen matrix in the tumor stroma as compared with the normal tissue, again suggesting the dissolution of the collagen matrix by collagenases [54-56]. The marked loss of fluorescence in the dermis surrounding the nonmelanoma skin tumor is probably due to the partial or total destruction of the collagen and elastin cross-links by fibroblast-derived collagenases. The destruction of collagen and elastin seems to occur primarily in the vicinity of the tumor [73]. The decrease in the fluorescence by basal cell carcinomas (BCC) is greater than that by squamous cell carcinomas (SCC), suggesting a more extensive invasion of the dermis by BCC than by SCC [73].

The decrease of collagen fluorescence can occur in the early stages of cancer development, before stromal invasion [75]. Changes in the scattering properties of the stroma can also occur in these very early stages [56]. Therefore, optical techniques, such as fluorescence spectroscopy, can be useful in probing the events occurring in the early stages of cancer development before the onset of tumor invasion.

### 5.1.8 Enzymatic Erosion of ex vivo Acellular Dermis to Mimic Melanoma Invasion

Our goal in this study is to determine if enzymatic treatment of skin dermis that mimics melanoma invasion can be detected using optical methods. Enzymatic cleavage of collagen in bovine tendon by pepsin has been used as a model for mimicking the early events in melanoma progression [76]. Second harmonic signals from pepsin-digested tendon have been shown to be significantly reduced relative to undigested tendon, suggesting a loss of fibrillar structure of the collagen matrix with pepsin treatment [76].
Although tendon, like dermis, is composed mostly of Type I collagen, collagen fibers in tendon are arranged parallel to one another, whereas collagen fibers in the dermis extend in many directions [4]. A second important difference between tendon and dermis is the presence of the basement membrane on the dermis. The presence of the basement membrane will subsequently affect the way in which enzymes interact with the dermal matrix.

As described before, the degradation of the basement membrane of skin is the initial step of melanoma invasion and metastasis, which is accomplished with the elevated expression of Type IV collagenases by melanoma and their adjacent stromal cells. After breaching the basement membrane, melanoma invades nearby dermis through elevated expression of Type I collagenase. Type I collagenase cannot cleave Type IV collagen, and therefore, cannot break down the basement membrane. Dispase has been used to remove the basement membrane from the dermis [77], since dispase cleaves Type IV collagen and fibronectin but only minimally degrades Type I collagen [78].

In this study, invasion of the dermis was modeled experimentally using an acellular dermis prepared from cadaver skin. Dispase was used in this study to break down the basement membrane on the dermis and to mimic the earliest step of melanoma invasion. To model later disruption to the matrix, dispase was used to completely remove the basement membrane, and then Type I collagenase was applied to the ex vivo acellular dermis to partially erode the matrix structure and mimic melanoma invasion.

The early events in tumor invasion are associated with a decrease in both collagen fluorescence and tissue scattering. The decrease in both collagen fluorescence and tissue scattering is suggested to be the result of the collagenase-catalyzed degradation of the
ECM surrounding the tumor. The extent of the decrease in collagen fluorescence and tissue scattering is associated with the stage of tumor progression. Fluorescence and reflectance spectroscopy were used to confirm that the enzymatic digestion of the dermal matrix leads to a decrease in fluorescence and scattering. To determine if fluorescence and reflectance spectroscopy are capable of detecting cumulative changes in degradation resulting from extended enzymatic digestion, samples of dermis were exposed to enzyme for different lengths of time and then spectra were collected. Extended exposure to enzyme may also mimic longer-term changes in matrix associated with tumor invasion and progression. Since enzymatic erosion of the dermis may also change the physicochemical properties of the collagen fluorophores and their microenvironment, the fluorescence anisotropy of the enzyme-treated tissues was also calculated and compared.

5.2 Materials and Methods

Enzymes were used to treat the dermis to mimic tumor invasion. Fluorescence and reflectance spectroscopy was used to investigate the fluorescence and scattering properties of both the papillary and reticular dermis sides of the dermis and to detect the changes in the enzyme-treated dermis.

5.2.1 Enzymatic Digestion of Acellular Dermis

De-epidermized dermis (DED) was prepared as described in Chapter 2. The DED was further processed by 10 cycles of freezing in liquid nitrogen and thawing in sterile deionized water at 37°C to destroy any cellular components in the dermis [79, 80]. The
DED, which was about 1mm thick, was cut with a sterile scalpel into small pieces with a size of about 10mm×20mm. For enzyme treatment, one piece of DED was transferred to each well of a six-well tissue culture plate (Greiner Labortechnik) with the papillary dermis side facing up. A sterile stainless steel ring with an inner diameter of 8mm was placed on one side of each piece of DED as shown in Figure 5.3. The ring was lightly pressed against the DED to form a non-leaking well over the tissue for enzyme solution addition.

Figure 5.3 Schematic representation of the treatment of DED with enzymes.

To determine the time required for dispase removal of the basement membrane, a 0.5ml aliquot of 1.2U/ml dispase (Sigma-Aldrich) in acetate buffer (1mM sodium acetate, 5mM calcium acetate, pH 7.50) was placed into the ring; the rest of the DED was submerged by addition of 3 ml of acetate buffer to the plate well (see in Figure 5-3). A total of 4 pieces of DED were prepared as described. Three DEDs were treated with 1.2U/ml dispase (Sigma-Aldrich) in acetate buffer (1mM sodium acetate, 5mM calcium acetate, pH7.50). The fourth was submerged with 3.5 ml of acetate buffer only as a control. The six-well tissue culture plate was then placed into a humidified 37°C cell
culture incubator (NAPCO). The dispase-treated DEDs were evaluated at incubation times of 1, 2, and 3 hours, and the control DED was evaluated after 3 hours of incubation. For each sample, the DED was taken out of the well, rinsed 3 times with sterile deionized water, and placed on a slide for spectroscopic measurements. Sterile deionized water drops were placed on edges of the DED sample to keep it moist while the spectra were collected. The DED samples were fixed in 10% formalin immediately after the spectroscopic measurements, and were sent to the Medical University of Ohio (MUO) for histology. The fixed DED samples were stained with periodic acid-Schiff staining (PAS staining) to detect the presence of the basement membrane \[77\]. The PAS stained tissue slides were examined under a microscope for the basement membrane structure.

The next set of experiments involved treating DED samples with dispase and Type I collagenase. A total of 8 pieces of DED were placed in the wells of two six-well tissue culture plates. Two of the DEDs were treated with acetate buffer only as a control. The remaining 6 DEDs were treated with dispase. One DED was evaluated spectroscopically after 1-hour incubation with dispase; all others were incubated for 2 hours as described above. After 2 hours of incubation, one DED treated with dispase and one DED control were removed for spectroscopy. The remaining 5 DEDs (4 treated with dispase, 1 control) were rinsed 3 times with Hank’s balanced salt solution (HBSS) buffer (pH7.50). A 0.5ml aliquot 100U/ml Type I collagenase (Sigma-Aldrich) solution in HBSS (pH 7.50) was added to the dispase-treated area on each of the 4 DEDs in the same way as for the dispase treatment. The plate well was then filled with 3 ml of HBSS to submerge the rest of the DED. The control DED was submerged with 3.5ml of HBSS buffer only as a control. The tissue culture plates were then placed back into the 37°C incubator. The
collagenase-treated DEDs were removed and evaluated spectroscopically after incubation times of 2, 4, 8, and 16 hours. The control DED was evaluated after 16 hours of incubation. The spectroscopic measurement procedure was the same as that described above for dispase treatment.

5.2.2 Fluorescence and Reflectance Spectroscopy of the DEDs

The experimental set-up for fluorescence and reflectance spectroscopy is the same as described in Chapter 3. The polarization orientation of the illumination light was set as parallel to the scattering plane. The collagen fluorophores that contribute to the fluorescence signal are complicated and largely unknown. Enzymatic digestion may cause different changes to the collagen fluorophores. Since the collagen fluorophores can be excited by light within the UVA region [41, 81, 82], three excitation wavelengths at 350, 360 and 375nm were used in this study to better characterize the complex nature of dermal fluorescence. These excitation wavelengths were provided by excitation bandpass filters (350nm±20nm, 360nm±20nm, and 375nm±20nm, Chroma Technology) within the excitation filter wheel.

Fluorescence and reflectance spectra were collected from 2 different sampling areas on each DED sample, one area being enzyme-treated and the other being an adjacent untreated control area. For the DEDs which were incubated with the buffers only, one sampling area was on one half of the DED and the other sampling area was on the other half. In addition to collecting spectra from the papillary dermis side, both fluorescence and reflectance spectra were also collected from the reticular dermis side of the control DEDs. Two non-polarized fluorescence spectra were collected at each excitation
wavelength from each sampling area on the DED. Immediately following collection of
the non-polarized fluorescence spectra, polarized fluorescence and polarized reflectance
spectra were collected from the sampling areas using the methods as described in Chapter
2 and Chapter 3. Fluorescence anisotropy and the degree of linear polarization (DLP)
were calculated using Equation 1.3 and Equation 1.2 as described in Chapter 1 and using
the methods as described in Chapter 2 and Chapter 3.

5.3 Results

Different fluorescence and scattering properties of the papillary and reticular dermis
sides of the DED were compared and discussed. The time required for basement
membrane removal with dispase was determined. The non-polarized fluorescence and
polarized reflectance spectroscopy of the enzyme-treated DEDs have confirmed that
enzymatic digestion of the dermal matrix leads to a decrease in fluorescence and
scattering. The fluorescence anisotropy of the DED, however, could not detect the
cumulative changes caused by enzymatic digestion. The results of studying DED
fluorescence emissions at multiple excitation wavelengths have shown that both the
fluorescence anisotropy and the enzyme-induced change in fluorescence intensity are
independent of the excitation wavelength.

5.3.1 Fluorescence and Reflectance of Dermis

To characterize the basic fluorescence and scattering properties of the dermis, non-
polarized fluorescence spectra, polarized fluorescence spectra, and polarized reflectance
spectra were obtained on both the papillary and reticular sides of 10 pieces of DED. The average fluorescence spectra of each DED sample was calculated by averaging the two measurements collected at each excitation wavelength on each sampling area. No photobleaching was observed when comparing the two measured fluorescence spectra from the same sampling areas. The average fluorescence spectra of all the samples were calculated by averaging the average fluorescence spectra of the 10 pieces of DED. The average non-polarized fluorescence spectra of all the DEDs with excitations at 350nm, 360nm, and 375nm are shown in Figure 5.4A-C. With all three excitation wavelengths, the reticular side of the DED yielded stronger fluorescence than the papillary side of the DED for emission wavelengths up to 600nm. The ratios of the average fluorescence spectra of the reticular side to the papillary side for the three excitation wavelengths are shown in Figure 5.4D. The fluorescence ratios are very close to one another for all three excitation wavelengths.

The average fluorescence spectra shown in Figure 5.4A-C were normalized using the peak fluorescence intensity within the spectra; the normalized spectra are shown in Figure 5.5A-C. To compare the effect of excitation wavelength on the emission spectra, the normalized spectra for the papillary dermis are shown in Figure 5.5D. To better view the spectral profile of the spectra shown in Figure 5.5, the effect of the wavelength-dependent response of the spectrometer was cancelled out by ratioing each average fluorescence spectra of each piece of DED to its average reflectance spectra (Figure 5.6). The resulting ratio spectra were then averaged over the 10 pieces of DED. The averaged ratio spectra was normalized against its peak ratio intensity, and the normalized ratio spectra for the papillary and reticular dermis sides are shown in Figure 5-7. The
normalized fluorescence/reflectance ratio spectra for the papillary dermis side of the DED are shown for the three excitation wavelengths in Figure 5.7D.

Figure 5.4 Comparison of the average fluorescence spectra of the papillary (solid lines) and reticular (dashed lines) sides of the DEDs excited at (A) 350nm, (B) 360nm, and (C) 375nm. The fluorescence spectra were averaged over the measurements of 10 pieces of DED. The integration time for spectra collection was 20ms for the 350nm excitation and 8ms for the 360 and 375 nm excitations. (D) The ratios of the average fluorescence spectra of the reticular side to the papillary side are nearly excitation wavelength independent. Dashed lines represent +/- one standard deviation for the data set.
Figure 5.5 Comparison of the normalized fluorescence spectra of the papillary (solid lines) and reticular (dashed lines) sides of the DEDs excited at (A) 350nm, (B) 360nm, and (C) 375nm. The fluorescence spectra were averaged over the measurements of 10 pieces of DED and normalized against their peak intensities. The normalized fluorescence spectra of the papillary side show a red-shift in emissions as the excitation wavelength increases (D).

Figure 5.6 Representative reflectance spectra collected from the papillary side of DED. The integration time for spectra collection was 20ms.
Figure 5.7 Comparison of the normalized ratio of fluorescence/reflectance spectra of the papillary (solid lines) and reticular (dashed lines) sides of the DEDs excited at (A) 350nm, (B) 360nm, and (C) 375nm. The non-polarized fluorescence spectra of each piece of DED were divided by the non-polarized reflectance spectra of the DED and the ratio spectra were averaged over the measurements of 10 pieces of DED. Each average ratio spectra was normalized against its peak intensity. (D) The normalized ratio of the fluorescence/reflectance spectra of the papillary side shows a red-shift in emissions as the excitation wavelength increases.

The normalized fluorescence/reflectance ratio spectra are increasingly red-shifted as the excitation wavelength increases, showing a fluorescence pattern typical of the collagen cross-links [14]. The red shift in emissions suggests that multiple fluorophores or multiple conformations of fluorescent macromolecules may exist in the collagen [34]. The normalized fluorescence/reflectance ratio spectra of the papillary side are also red-shifted as compared to those of the reticular side at the same excitation wavelengths.
Zeng et al. also found that the position of the fluorescence maximum of the papillary dermis was red-shifted by 20nm as compared with the reticular dermis for 442nm excitation, and suggested that different fluorophores might exist in these two dermal layers [34].

To compare the fluorescence anisotropy and light scattering of the papillary and reticular dermis, polarized fluorescence and polarized reflectance spectra were collected from both the papillary and reticular sides of the DEDs. The polarized fluorescence spectra were collected as described in Chapter 4. Briefly, sequential polarized fluorescence spectra were collected with the polarization orientation between the polarizer and analyzer set as parallel, perpendicular, perpendicular, and parallel. No photobleaching was observed when comparing the two parallel polarized fluorescence spectra. The polarized reflectance spectra were collected in the same manner immediately following the collection of the polarized fluorescence spectra and switch of the light source from the mercury light to the halogen light. The fluorescence anisotropy and degree of linear polarization (DLP) were calculated from the polarized fluorescence and polarized reflectance spectra, respectively, with the methods described in Chapter 4.

Representative polarized fluorescence and polarized reflectance spectra are shown in Figure 5.8. As with unpolarized emissions, polarized fluorescence emissions of the reticular side are stronger than those of the papillary side. To investigate the rotation ability of the collagen fluorophores, which may reflect the physicochemical properties and microenvironment of the fluorophores within the papillary and reticular dermis, fluorescence anisotropies were calculated for each DED sample and averaged over the 10 pieces of DED. The average fluorescence anisotropies are shown in Figure 5.9. The
fluorescence anisotropies are nearly independent of excitation wavelength. As shown in Figure 5.10A–C, the fluorescence anisotropies of the papillary side cannot be separated from those of the reticular side. To compare the light scattering within the papillary and reticular dermis, the DLP was calculated and averaged over the 10 pieces of DED. As shown in Figure 5.10D, the DLP of the backscattered light on the reticular side is appreciably higher than that on the papillary side.

Figure 5.8 Representative polarized fluorescence spectra of the papillary (magenta lines) and reticular (blue lines) sides of the DEDs excited at (A) 350nm, (B) 360nm, and (C) 375nm. The integration time was 150ms for excitation at 350nm and 40ms for the 360 and 375nm excitation wavelengths. (D) The corresponding polarized reflectance spectra are shown. The integration time for the polarized reflectance spectra was 80ms.
Figure 5.9 Fluorescence anisotropies calculated for each DED were averaged over the measurements of all 10 samples. Shown above are the average fluorescence anisotropies of (A) the papillary side and (B) the reticular side excited at 350nm (blue), 360nm (magenta), and 375nm (black). Dashed lines represent +/- one standard deviation for the data set.
Figure 5.10 Comparison of the average fluorescence anisotropies of the DEDs on the papillary side (blue lines) and the reticular side (magenta lines) excited at (A) 350nm, (B) 360nm, and (C) 375nm. The fluorescence anisotropies were averaged over the measurements of 10 pieces of DED. No appreciable difference is seen between the two sides. (D) The average degree of linear polarization (DLP) of the backscattered light from both sides is shown. The DLP was calculated for each sample and then averaged over the 10 pieces of DED. The DLP on the reticular side (magenta line) is appreciably higher than that on the papillary side (blue line). Dashed lines represent +/- one standard deviation for the data set.

In summary, the fluorescence emission of the reticular side of DED is stronger than that of the papillary side. This difference in fluorescence intensity may suggest that more fluorophores or fluorophores with higher quantum yield are present in the reticular dermis than in the papillary dermis. The appreciably higher DLP with the reticular side of the DED may suggest less scattering (lower density) in the reticular dermis than in the papillary dermis. However, the possible change of the matrix density of the reticular side of the DEDs caused by mechanic forces when tearing the skin off may also account for the lower DLP on the reticular side of the DEDs. No appreciable difference of the fluorescence anisotropy exists as a function of excitation wavelength or the side (papillary or reticular) of the DED.

5.3.2 Basement Membrane Removal by Dispase Treatment

Three pieces of DED were treated with dispase; a DED sample was removed and evaluated spectroscopically after 1hr, 2 hrs, and 3 hrs of incubation. A control DED treated with buffer only was removed and spectroscopically evaluated after a 3 hrs incubation. The non-polarized fluorescence spectra of the four samples generated using excitation wavelengths of 350nm, 360nm and 375nm were collected to see if the effects of dispase treatment could be detected. Two spectra were collected from each sampling
Figure 5.11 Fluorescence spectra of the DED samples for the (A-C) control, (D-F) 1 hr dispase treatment, (G-I) 2 hr dispase treatment, and (J-L) 3 hr dispase treatment. The excitation wavelengths used are by column at (A, D, G, J) 350nm, (B, E, H, K) 360nm, and (C, F, I, L) 375nm. After 2 hrs of dispase treatment, the fluorescence intensities of the enzyme-treated areas are appreciably lower.
than the control areas at all 3 excitation wavelengths. The integration times are 10ms for the 350nm excitation and 3ms for the 360nm and 375nm excitation wavelengths.

area and averaged. The average spectra for each area on the four samples are shown in Figure 5.11. No photobleaching was detected. For each excitation wavelength, no difference was seen in the fluorescence spectra of the two sampling areas on the control DED (Figure 5.11A-C). After 1 hr of dispase treatment, the fluorescence intensities of the control and treated areas show a small deviation for 350nm, but no detectable difference for the 360 and 375nm excitation. However, by 2 hrs, the fluorescence intensities of the dispase-treated areas are noticeably lower than for the control areas at all excitation wavelengths.

Ratios of the fluorescence spectra in the dispase treated area to the control area were calculated for each DED sample. For the control DED, the fluorescence ratio spectra was calculated by ratioing the fluorescence spectra of the two adjacent sampling areas. The fluorescence spectra ratios are shown in Figure 5.12. The fluorescence intensities of the two sampling areas on the control DED show small variations at both ends of the spectra, which are not noticeable in the raw spectra (Figure 5.11A-C). After 1 hr of dispase treatment, the fluorescence intensity of the dispase treated area is little lower than for the control area with excitation at 360nm but is apparently higher than for the control area within the lower emission wavelength region excited at 375nm. Both the changes are not noticeable in the raw spectra (Figure 5.11E, F), indicating that the fluorescence spectra ratio is more sensitive in detecting spectral changes than the raw fluorescence spectra. These changes of fluorescence intensity could be due to the spatial variation of fluorophores within the DEDs. After 2 hrs of dispase treatment, the decrease in the fluorescence intensity as seen in the ratios is about the same for all 3 excitation
wavelengths. No additional decrease in the fluorescence intensity is seen in the 3 hr treatment with dispase. There are sample-dependent variations of fluorescence intensity between the DEDs as demonstrated with the variations of fluorescence intensity in the control areas on different DEDs. The dependence of fluorescence on body location has also been reported [33].

Figure 5.12 Fluorescence spectra ratios between dispase treated and control regions on each DED sample. For the control sample, the ratio is between two adjacent sampling areas. Panels show the ratios for the (A) control, (B) 1hr, (C) 2hr, and (D) 3 hr dispase treatments with excitation at 350nm (blue solid line), 360nm (magenta dashed line), and 375nm (green dotted line).

The representative results of the PAS staining of the dispase-treated DEDs are shown in Figure 5.13. Under the experimental conditions used in this study, the PAS staining
shows remnants of the basement membrane for the 1 hr dispase treatment. However, the basement membrane was totally removed from the DED after 2 hrs of dispase treatment. The trend observed with the fluorescence spectra ratios is consistent with these PAS staining results.

Figure 5.13 Representative microscopic images (40×) of the PAS staining of the DEDs for (A) 1 hr dispase treatment (B) 2 hr control area, and (C) 2 hr dispase treatment area. Remnants of the basement membrane structure can still be seen on the 1 hour-treated area. For the 2 hr treatment, continuous basement membrane (BM) structure is seen on the DED control, whereas the basement membrane structure is absent in the dispase treated area. Images were taken in Dr. Akkus’ laboratory.

5.3.3 Enzymatic Digestion of the Dermal Collagen by Dispase and Collagenase

Non-polarized fluorescence, polarized fluorescence, and polarized reflectance spectra were collected from enzyme-treated DEDs. The non-polarized fluorescence spectra were used to calculate the fluorescence spectra ratio of the treated to the control areas on the DED to compare the changes in both spectral profile and intensity. Integrated fluorescence intensity was also calculated from the non-polarized fluorescence spectra to quantify the change in the fluorescence emissions between the treated and control areas on the DED. Fluorescence anisotropy of the DED was calculated from the polarized
fluorescence spectra to investigate the effect of enzymatic digestion on the rotation ability of the collagen fluorophores within the DED. The DLP was calculated from the polarized reflectance spectra to study the effect of enzymatic digestion on the scattering within the enzyme-treated DED.

5.3.3.1 Fluorescence Emission Spectra of Dispase and Collagenase Treated DEDs

Non-polarized fluorescence spectra were used to study the dynamic change of the dermal matrix induced by enzymatic erosion. Each DED was treated with enzyme solution for differing times and the fluorescence emission spectra for both the enzyme-treated area and its adjacent control area on the DED were collected. As a reference, non-polarized fluorescence spectra were also collected from two sampling areas on the DED controls. The fluorescence measurements were repeated on 9 batches of DEDs on different days. Representative fluorescence spectra of the individual DED excited at 360nm are shown in Figure 5.14. The fluorescence spectra were averaged over two measurements taken on each sampling area; no photobleaching was observed. The fluorescence intensities of the enzyme-treated areas at all 3 excitation wavelengths decreased relative to the adjacent control areas for treatment times greater than 2 hrs. Sample-dependent variations of fluorescence intensity are apparently seen in Figure 5.14, whereas spatial changes of fluorescence intensity are minimal on the DED samples with a size of 10mm×20mm, as can be seen from the fluorescence spectra of the two control DEDs (Figure 5.14).
Figure 5.14 Representative fluorescence spectra of the DEDs for (A) control for dispase treatment; treatment with dispase for (B) 1 hr and (C) 2 hrs; (D) control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for (E) 2 hrs, (F) 4 hrs, (G) 8 hrs, and (H) 16 hrs. The control spectra shown are for the control (A) incubated in the acetate buffer for 2 hrs, and (D) incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). The fluorescence spectra were collected with excitation at 360 nm and with an integration time of 8 ms. The fluorescence spectra of the enzyme-treated areas (blue solid line) show a decrease in fluorescence intensity relative to the adjacent control areas (magenta dashed line) for total treatment time greater than 2 hrs.
Figure 5.15 Average fluorescence spectra ratios of the DEDs for (A) control for dispase treatment; treatment with dispase for (B) 1hr and (C) 2 hrs; (D) control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for (E) 2 hrs, (F) 4 hrs, (G) 8 hrs, and (H) 16 hrs. The control spectra shown are for the control (A) incubated in the acetate buffer for 2 hrs, and (D) incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). Averages were calculated from the fluorescence spectra ratios from either 8 or 9 DEDs as indicated in the panel legends. Dashed lines represent +/- one standard deviation for the data set.
Fluorescence spectra ratios were calculated as described in Section 5.3.2 by ratioing the treatment area spectrum to the control area spectrum. The spectra ratios calculated for the 9 DEDs were averaged. As shown in Figure 5.15, enzyme-induced fluorescence changes appear to be independent of the excitation wavelength. The average fluorescence spectra ratios of the enzyme-treated DEDs for 360 nm excitation are replotted in Figure 5.16 to show the effect of treatment time. The fluorescence spectra ratios of the two control DEDs are very flat and equal to unity, showing the reproducibility of the spectra generated within a sample. After a 1 hr treatment with dispase, the fluorescence spectra ratio is still flat but has decreased slightly. After treatment with dispase for 2 hrs, the fluorescence spectra ratio is not longer flat, but decreases considerably with increasing emission wavelength to a value near 0.85. For all subsequent treatments with collagenase, the fluorescence spectra ratios are basically unchanged regardless of the collagenase treatment time.
Figure 5.17 The average ratio of total fluorescence intensity is shown as a function of total enzyme treatment time (either dispase or dispase and collagenase). Total fluorescence intensity ratios were calculated from the fluorescence spectra of the DEDs excited at 350nm (blue star), 360nm (magenta triangle), and 375nm (black circle). Data show the means and standard deviations for 8 DEDs. After 2hrs of treatment with dispase, the total fluorescence intensities at all 3 excitation wavelengths significantly decreased as compared to the control (p<0.001) and 1hr treatment with dispase (p<0.05). No significant decrease in the total fluorescence intensities was observed between the control and 1hr treatment with dispase.

To quantify the change in the fluorescence emissions between the treated and control areas on each sample, the total fluorescence generated in each area was calculated and ratioed. The total fluorescence for a sampling area was calculated from the fluorescence emission spectrum, which was the average of 2 measurements collected, by integrating over the entire spectral range collected from 420nm to 600nm. The ratio of the total fluorescence in the treatment area to the control area on each DED was then calculated. The average ratios of total fluorescence intensity were calculated from the 8 DEDs in each treatment group. The average ratios of fluorescence intensity are shown in Figure 5.17 as a function of total enzyme treatment time (either dispase or dispase and collagenase). A sharp decrease of the average ratio of fluorescence intensity occurred during the first 2 hrs of treatment with dispase. The subsequent treatments with
collagenase did not cause any additional decrease in the fluorescence intensity ratio. The enzyme-induced changes in the average ratio of total fluorescence intensity are very similar for all 3 excitation wavelengths (see Figure 5.17).

5.3.3.2 Fluorescence Anisotropy of Dispase and Collagenase Treated Dermis

To investigate if enzymatic erosion changes the physicochemical properties of the collagen fluorophores and their microenvironments, polarized fluorescence spectra were collected on the same enzyme-treated DEDs as discussed previously in this Section 5.3.3. As presented in Section 5.3.1, the fluorescence anisotropies of the native, untreated DEDs are almost identical for all 3 excitation wavelengths (see Figure 5.9). To determine if enzymatic treatment results in excitation wavelength dependent changes in the fluorescence anisotropy, polarized fluorescence emissions spectra were collected from the enzyme-treated DEDs. The fluorescence anisotropy for each DED was calculated at each excitation wavelength. The fluorescence anisotropies were averaged over the 9 DEDs and the average fluorescence anisotropies are shown in Figure 5.18. No appreciable excitation wavelength dependent differences in the fluorescence anisotropies were observed on the enzyme-treated DEDs. Considering that no difference is detected between fluorescence anisotropy from the 3 excitation wavelengths, the results suggest that the excitation wavelengths seem to target the same fluorophores, or the enzymes non-differentially digest all the fluorophores which are excited with these 3 excitation wavelengths.

The average fluorescence anisotropies of the enzyme-treated and control areas for each treatment condition and for the DED controls are shown in Figure 5.19. The average
fluorescence anisotropies are calculated from the fluorescence anisotropies for either 8 control or 9 treatment DED samples; results are shown for 360nm excitation. For all the treatment conditions tested, no significant differences are observed between the treated and control areas. Thus, it appears that the enzyme digestion of the matrix did not change the rotation ability of the collagen fluorophores.

Figure 5.18 Average fluorescence anisotropies of the DEDs as a function of excitation wavelength for (A) control for dispase treatment; treatment with dispase for (B) 1hr and (C) 2 hrs; (D) control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for (E) 2 hrs, (F)
4 hrs, (G) 8 hrs, and (H) 16 hrs. The control spectra shown are for the control (A) incubated in the acetate buffer for 2 hrs, and (D) incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). Averages were calculated from the fluorescence anisotropy calculated from either 8 or 9 DEDs as indicated in the panel legends. Dashed lines represent +/- one standard deviation for the data set. The excitation wavelengths used were 350nm (blue), 360nm (magenta), and 375nm (black).

Figure 5.19 Comparison of the average fluorescence anisotropy of the enzyme-treated areas (blue curves) with that of the control areas (magenta curves). Average fluorescence anisotropies of the DEDs for an excitation wavelength of 360 nm are shown for (A) control for dispase treatment; treatment with dispase for (B) 1hr and (C) 2 hrs; (D) control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for (E) 2 hrs, (F) 4 hrs, (G) 8 hrs, and (H) 16 hrs. The control spectra shown are for the control (A) incubated in the acetate buffer for 2 hrs, and (D) incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). Averages were calculated from the fluorescence anisotropy calculated from 8 control or 9 treatment DEDs. Dashed lines represent +/- one standard deviation for the data set.
5.3.3.3 Dispase and Collagenase Treated Dermis Probed with the Degree of Linear Polarization (DLP) of the Backscattered Light

To investigate whether enzymatic erosion changes the scattering properties of the dermal matrix, polarized reflectance spectra were collected from all of the DEDs treated with dispase and collagenase as previously described. Typical polarized reflectance spectra of the DEDs treated with the enzymes for differing times are shown in Figure 5.20. The general shape of the polarized reflectance spectra is unchanged by enzymatic treatment. The parallel and perpendicular polarized reflectance spectra were averaged over the two measured spectra on each DED, respectively, and the DLP was then calculated by dividing the difference between the average parallel and perpendicular polarized reflectance spectra by the sum of these two spectra. The DLPs were calculated from the spectra collected on 9 DEDs and averaged. The average DLPs of the backscattered light from the enzyme-treated areas and control areas are shown in Figure 5.21. The average DLPs of the backscattered light from the control and the enzyme-treated areas on the DEDs are nearly identical for up to 4 hrs of total enzyme treatment. However, after 6hrs of enzyme treatment (2 hrs of dispase followed by 4 hrs of collagenase), the average DLP of the backscattered light from the enzyme-treated areas increased appreciably relative to the control areas. The average DLP continued to increase with increased collagenase incubation. The average DLPs in the DEDs for all treatment areas and all control areas are replotted in Figure 5.22. As the DLP increases, the dermal scattering decreases. No change in dermal scattering is seen in any of the control areas. In addition, no change in dermal scattering is seen with basement membrane degradation by dispase. However, dermal scattering decreases with increasing
time of collagenase digestion, indicating a loss of scattering structures, most likely collagen fibers, in the dermis.

Figure 5.20 Representative polarized reflectance spectra of the DEDs are shown for (A) control for dispase treatment; treatment with dispase for (B) 1 hr and (C) 2 hrs; (D) control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for (E) 2 hrs, (F) 4 hrs, (G) 8 hrs, and (H) 16 hrs. The control spectra shown are for the control (A) incubated in the acetate buffer for 2 hrs, and (D) incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). The polarized reflectance spectra were averaged from the two spectra collected in either the control area (magenta) or treatment area (blue) on the enzyme-treated DEDs. For the control DEDs, the polarized reflectance spectra were averaged from the two spectra collected in either of the two sampling areas (magenta, or blue). The polarized reflectance spectra were collected...
with an integration time of 80ms except the DED treated with dispase for 2 hrs was integrated for 70ms.

Figure 5.21 Comparison of the average DLP of the backscattered light from the enzyme-treated areas (blue) with that from the control areas (magenta) for (A) control for dispase treatment; treatment with dispase for (B) 1hr and (C) 2 hrs; (D) control for collagenase treatment; and 2 hr dispase treatment followed by collagenase treatment for (E) 2 hrs, (F) 4 hrs, (G) 8 hrs, and (H) 16 hrs. The average DLP in the DED controls shown are for the control (A) incubated in the acetate buffer for 2 hrs, and (D) incubated in the acetate buffer for 2 hrs, followed by incubation in HBSS for 16 hrs (See methods). The average DLP values were calculated from the DLPs of either 8 or 9 DEDs as specified in the panel legends.
5.4 Discussion

Previous studies have revealed that a decrease in fluorescence emission and tissue scattering is associated with tissue malignancy in various tissue types, including skin. Collagenase-induced dissolution of the ECM has been suggested to be the cause of the reduction of the fluorescence emission and scattering of the malignant tissue. In this study, enzymatic erosion of the dermal extracellular matrix (DED) was employed to mimic the breakdown of the dermal matrix during melanoma progression.

Fluorescence and reflectance spectroscopy were used to detect the enzyme-induced changes of the DED. Due to the complex nature of the fluorophores in the dermis, the excitation wavelengths of 350nm, 360nm, and 375nm were used in this study. The fluorescence spectra of the DED excited at these 3 wavelengths were red-shifted with increasing excitation wavelength, suggesting that multiple types of fluorophores or multiple conformations of a single fluorophore may exist in the dermis. However, enzymatic degradation of the DED, either by dispase to remove the basement membrane...
or by the collagenase to break down the Type I collagen structure, did not show any excitation wavelength-dependent changes in the fluorescence emission. The 3 excitation wavelengths may target the same fluorophores, which could be the collagenase-digestible collagen cross-links [41-43]. Alternately, the actions of dispase and collagenase may have no specificity for any of the different types of fluorescent cross-links in the dermis. Collagenases specifically cleave some peptide bonds on the \( \alpha \) chains of the collagen molecule but not the cross-links of the collagen fibril [4]. The resulting collagen fragments spontaneously denature at temperatures greater than 33°C [4]. Therefore, the cleavage of the dermal matrix by the collagenases may lead to a uniform degradation of all the various fluorophores in the dermis.

The fluorescence anisotropies of the DED excited at the 3 excitation wavelengths did not show any excitation wavelength dependency. This, again, may be due to the same fluorophores being excited by all 3 excitation wavelengths, or to the different fluorophores having similar rotational diffusion rates. The fluorescence anisotropies of the DEDs did not show any changes with enzyme-treatment at any of the excitation wavelengths. It appears that the enzyme treatments did not change the physicochemical properties of the remaining fluorophores and their microenvironment but simply destroyed the fluorophores. It can be concluded from these results that the fluorescence measurements of the enzyme-treated DEDs with the excitations at 350nm, 360nm, and 375nm provide redundant information.

Enzymatic erosion of the DED appreciably decreased the fluorescence emission relative to the untreated control in this study, especially during the first 2 hrs of incubation with dispase. Extended treatment of the DED with dispase or dispase
treatment followed by treatment with collagenase did not result in a further decrease in the fluorescence emissions. Dispase specifically cleaves Type IV collagen but only minimally degrades Type I collagen [78]. The results in this study show that the basement membrane structure, namely Type IV collagen-rich lamina densa, was totally removed by 2 hr incubation with dispase. Thus, the sharp decrease in the fluorescence emission during the first 2 hrs of treatment with dispase was most likely due to the degradation and destruction of the fluorophores within the lamina densa. Why did the subsequent treatment with collagenase not cause a further decrease in the fluorescence emission? Several explanations are possible. One explanation is that the collagenase destroyed the fluorophores in the papillary dermis by removing this part of the dermis gradually through the prolonged incubation with the collagenase. Since the papillary dermis is considerably thinner (about 100μm) and has lower fluorescence than the reticular dermis as demonstrated with the results in this study, the removal of the papillary dermis may lead to excitation of more fluorophores in the reticular dermis, and thus, stronger backscattered fluorescence emissions which might counteract the reduction in the fluorescence emission due to the erosion of the papillary dermis. Another explanation is that the collagenase cleaves the specific peptide bonds on the α chains of the collagen molecule but not the cross-links of the collagen fibril. Thus, collagen monomers might be cleaved but the fragments would still be held in place by the cross-links [4]. As a result, the fluorophores may survive the enzymatic digestion. The first explanation seems more reasonable, since some pieces of the DED incubated with collagenase for long times (i.e. 16 hours) showed that the enzyme-treated areas were eroded a very little bit.
The polarized reflectance is not as sensitive as the fluorescence emission in detecting the early changes of the DED resulting from enzymatic treatment. The DLP in the enzyme-treated DED only increased appreciably relative to the control after 2 hr of dispase treatment followed by 4 hr of collagenase treatment. The DLP in the enzyme-treated DED increased further with the prolonged incubation with collagenase. In addition to scattering, linear birefringence in the dermis also contributes to the depolarization of the initially polarized incident light [51]. Enzymatic erosion destroys the scattering and birefringent structures, namely the collagen fibers, and more collagen fibers are destroyed during the prolonged treatment with collagenase, thus leading to a higher DLP in the enzyme-treated DED. As demonstrated in this study, the papillary dermis has stronger scattering than the reticular dermis as the measured DLP is lower in the papillary dermis than the reticular dermis. In addition, since the reticular dermis is less scattering than the papillary dermis, loss of the papillary dermis by enzyme treatment may also contribute to the increase in DLP. As the papillary dermis is gradually removed through the incubation with collagenase, the reticular dermis is gradually exposed to the initially polarized incident light, leading to an increase in DLP as a result of less scatter in the reticular dermis. However, the enzymatic degradation of the collagen fibers of the dermal matrix might be the major cause, since the DLP in the enzyme-treat DED increased further with the prolonged incubation with collagenase.

In summary, the structural changes of the DED induced by the enzymes can be monitored with fluorescence emission spectroscopy and polarized reflectance spectroscopy. In this study, both the fluorescence emission and the scattering of the DED decreased with enzymatic erosion. These results support the literature findings with in
\textit{vivo} and ex \textit{vivo} tissues that tissue malignancy leads to decreased fluorescence emission and scattering in the tumor stroma. The results in this study also confirm that the changes of the fluorescence emission and scattering of the stroma are consistent with the enzymatic cleavage of the collagen fibers in the ECM. Under the conditions in this study, the fluorescence emission intensity was very sensitive to the early structural changes of the DED as the basement membrane was being removed by dispase. However, the dispase-treated DED did not show any change in DLP. Conversely, the DLP increased as the collagenase destroyed the underlying Type I collagen, but the fluorescence emission intensity did not change during this period of prolonged incubation. Fluorescence measurements of the DED with multiple excitation wavelengths between 350nm and 375nm appear redundant, and the fluorescence anisotropy of the DED did not change with the enzyme treatment. Therefore, the enzymatic cleavage of the dermal collagen seems to non-differentially destroy the various fluorophores in the dermis due to the action mechanism of the collagenase, and the enzymatic cleavage does not change the physicochemical properties of the remaining fluorophores and their microenvironment. Thus, combined fluorescence emission and polarized reflectance spectroscopy can thus be used to probe the enzyme-induced structural changes of the ECM.

5.5 Future Work

Since the degradation of the ECM by tumor invasion is caused by the cleavage of the collagen matrix with the concerted action of multiple enzymes, a more realistic model would include more degradation enzymes to better mimic melanoma invasion. The model developed would use a multi-enzyme system (Type I collagenase + elastase + neutral...
proteinase [4]) with the DED. This model would better mimic the structural and thus optical changes occurring in the tissue and would provide insight into the fluorescence emission with the enzyme treatment time observed in this study since the fluorophores may survive the treatment with only the collagenase. Since DLP is not sensitive to the early structural changes of the DED induced by the enzyme, other reflectance techniques, such as the measurement of the scattering coefficient, would be helpful in probing the early structural changes of the enzyme-treated DED.

Reference:


Chapter 6

General Conclusions and Future Suggestions

To answer the questions raised at the beginning of this dissertation, experiments were designed and conducted using polarized fluorescence spectroscopy. Polarized reflectance, unpolarized fluorescence, and unpolarized reflectance spectroscopy were also used in some studies where the problems merited a study with these additional modes to contribute to understanding of the results obtained. The general findings are summarized in the following paragraphs.

As shown in Chapter 3, polarized fluorescence emissions can be generated from the epidermis, the dermis, and the skin using polarized excitation light. The epidermis has the highest retention of fluorescence polarization. As the thickness of the dermis increases, the depolarization of fluorescence increases and the fluorescence anisotropy decreases. This result supports the premise that multiple scattering within the dermis contributes to the depolarization of fluorescence within the skin. There is residual fluorescence anisotropy and a residual degree of linear polarization in the dermis, which are relatively independent of dermal thickness when the dermal thickness is greater than a few hundred microns. Since the dermis in intact skin is about 1 to 4mm thick, this implies that changes found in the fluorescence anisotropy and the degree of linear polarization in the skin are more likely caused by the structural changes (i.e. density) within the tissue than from variation in the skin thickness. These results were obtained on skin and skin layers
obtained from cadaveric sources that were maintained in unsupplemented PBS. It is unlikely that any cells remained viable when these measurements were made, and hence it is unlikely that fluorophores associated with metabolism are present in conditions that are representative of living tissue. Thus, the fluorescence and polarization properties obtained from in situ human skin will vary to some degree from those presented here.

Another interesting observation in the study of the dependence of the fluorescence anisotropy and degree of linear polarization (DLP) on dermal thickness is related to the orientation of the excitation light and the scattering plane. When the polarization orientation of the excitation light was changed from parallel to perpendicular to the scattering plane, the fluorescence anisotropy of the thick dermis (thickness > 150µm) changed from positive to negative. However, with the same dermis samples, when the polarization orientation of the illumination light was switched between parallel and perpendicular to the scattering plane for the reflectance spectroscopy measurements, the calculated DLP did not show a similar change. The reason for this change with fluorescence anisotropy is unknown.

Changes in cellular NADH associated with metabolic changes in cells can be studied with polarized fluorescence spectroscopy as was shown in experiments in Chapter 4 using cell suspensions. As expected, the melanoma cells have significantly lower fluorescence anisotropy than the normal melanocytes, most likely due to less NADH-protein binding and/or a less stringent microenvironment for NADH in the cancer cells. These changes are assumed to be caused by the metabolic shift of the cancer cells to enhanced glycolysis. The metabolic shift of cancer cells from aerobic respiration to enhanced glycolysis is a near universal phenomenon of cancers. This fluorescence
technique may thus find wide application in the in situ detection of various cancers. Additional applications include the real-time tracking of changes in metabolism of cells as a result of oxygenation or drug administration.

Finally, experiments were performed on a de-epidermized dermis (DED) to determine if degradation of the dermal matrix could be detected using fluorescence techniques. The fluorescence associated with the DED is associated with tissue structure only as no cellular components (and hence no NADH) remain. The results from fluorescence measurements of the DED with multiple excitation wavelengths between 350nm and 375nm appear redundant. When the DED was degraded with dispase followed by collagenase, the fluorescence anisotropy of the DED did not change relative to the control. However, the fluorescence emission intensity decreased appreciably, while the degree of linear polarization (DLP) increased appreciably with enzyme degradation relative to the control. The fluorescence emission intensity and the DLP address difference stages of dermal degradation. The fluorescence emission intensity is very sensitive to the early structural changes of the DED as the basement membrane is being removed by dispase. As the collagenase destroys the underlying Type I collagen, no further change in fluorescence intensity is observed. Conversely, the DLP shows no changes in the early stages of matrix degradation with dispase, but instead increases as the collagenase destroys the underlying Type I collagen. These changes suggest that dispase treatment results in the loss of fluorophores from the matrix. The extended treatment with collagenase and the scission of the \( \alpha \) chains in collagen likely creates a less scattering matrix without the loss of fluorophores from the matrix.
The completion of this dissertation provides the basis for several areas of future study. The first topic is to increase the fluorescence polarization of the backscattered fluorescence emissions by designing a new fiber probe. When comparing the results of the fluorescence anisotropy measurements in Chapters 2 and 5, one can see that the fluorescence anisotropy of the dermis increased from 0.018 to 0.035 (excitation/emission at 360nm/420nm) when the diameter of the circular illuminating area was decreased from about 0.9cm to about 0.5cm. This means that by decreasing the illumination diameter by half and thus increasing the excitation light density, the fluorescence anisotropy nearly doubled. An increase in the retained fluorescence polarization (fluorescence anisotropy) translates into an enhancement of the signal to noise ratio associated with backscattered fluorescence originating in the superficial tissue versus deeper tissue sites.

Another suggestion is the investigation of whole tissue with fluorescence anisotropy measurements. NADH and collagen contribute to most of the autofluorescence of the epithelial tissue at excitation wavelengths within the UVA range. The total fluorescence anisotropy of the epithelial tissue which results from the combination of fluorescence generated from each of the fluorophores can thus be calculated as

\[ r = r_{NADH}f_{NADH} + r_{collagen}f_{collagen} \]  \[1\]

where \( r \) is the fluorescence anisotropy of the tissue; \( r_{NADH} \) and \( r_{collagen} \) are the fluorescence anisotropy of the NADH and collagen, respectively; and \( f_{NADH} \) and \( f_{collagen} \) are the portion of fluorescence intensity for the NADH and collagen, respectively. According to the results obtained in this dissertation, when the degradation of the ECM occurs, \( r_{collagen} \) does not change but is equal to that of the normal ECM, whereas \( f_{collagen} \) decreases appreciably. The fluorescence anisotropy from NADH, \( r_{NADH} \) decreases
appreciably due to the metabolic shift of cancer cells. However, $f_{\text{NADH}}$ may not change appreciably with tissue malignancy, and $f_{\text{NADH}}$ is much lower than $f_{\text{collagen}}$ as reported in the literature. Therefore, the measured $r$ for the tissue may be more dependent on the changes of $f_{\text{collagen}}$ and $r_{\text{NADH}}$, yielding an appreciably lower value with tissue malignancy. Fluorescence anisotropy from both malignant and benign tissue samples should be investigated to test this hypothesis.

Reference: